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# CONTENTS

No. 1—January

	PAGE
DAVIDSON, J. N., I. LESLIE and J. C. WHITE, The cytoplasmic biophilin of marrow cells: the distribution of nucleic acids (plates I-IV) . . .	1
STEWART, M. J., and GEORGINA M. BONSER, Melanin-forming epidermal tumours of the skin: a study of 57 personally observed cases (plates V-VIII) . . . . .	21
TAYLOR, JOAN, WILLIAM HAYES, JOSEPH FREEMAN and E. S. ANDERSON, A new <i>Salmonella</i> type: <i>Salmonella chittagong</i> . . . . .	35
ROBERTSON, J. S., and K. VINER SMITH, An analysis of certain factors associated with the production of experimental dissection of the aortic media, in relation to the pathogenesis of dissecting aneurysm . . . . .	43
DAVIDSON, JANE I., Massive colony formation of <i>Bacterium friedländeri</i> in the liver in agranulocytosis (plates IX and X) . . . . .	51
DUGUID, J. B., Thrombosis as a factor in the pathogenesis of aortic atherosclerosis (plates XI-XVI) . . . . .	57
COLLINS, DOUGLAS H., and W. McI. ROSE, The nature of anaemia in leukaemia (plates XVII and XVIII) . . . . .	63
POPJÁK, G., The mechanism of parenchymatous degeneration produced by diphtheria toxin (plates XIX-XXI) . . . . .	75
BISSET, K. A., The effect of temperature upon antibody production in cold-blooded vertebrates . . . . .	87
MACCALLUM, P., JEAN C. TOLHURST, GLEN BUCKLE, and H. A. SISSONS, A new mycobacterial infection in man (plates XXII-XXIX) . . . . .	93
MACCALLUM, P., I. Clinical aspects . . . . .	93
TOLHURST, JEAN C., and GLEN BUCKLE, II. Experimental investigations in laboratory animals . . . . .	102
SISSONS, H. A., III. Pathology of the experimental lesions in the rat . . . . .	110
BUCKLE, GLEN, and JEAN C. TOLHURST, IV. Cultivation of the new mycobacterium . . . . .	116
EVANS, R. WINSTON, The antibody response in cases of radiation lymphopenia, and in the reticuloses . . . . .	123
SVENDSEN, MAGNE, Leucocytes and bacteria in human semen . . . . .	131

	PAGE
ISAACS, ALICK, and J. M. SCOULLER, Catalase production by Gram-positive cocci: a simple test for differentiating enterococci from micrococci . . . . .	135
HENDERSON, W. M., Further consideration of some of the factors concerned in intracutaneous injection of cattle . . . . .	137
THOMSON, J. G., Haemolysis in fresh blood films . . . . .	140
BOOKS RECEIVED . . . . .	142
PROCEEDINGS OF THE SOCIETY . . . . .	147
ABUL-FADL, M. A. M., and E. J. KING, The application of formaldehyde to the determination of serum acid phosphatase . . . . .	149
DAY, T. D., The spread of fluids in connective tissue . . . . .	150
ROBINSON, ALICE M., and F. L. WARREN, The use of polarographic analysis in the investigation of pathological sera . . . . .	152
REWELL, R. E., Sarcoma of the tibia in a coypu rat ( <i>Myocastor coypus</i> ) . . . . .	154
REWELL, R. E., Seminoma of the testis in a collared turtle dove ( <i>Streptopelia risoria</i> ) . . . . .	155
REWELL, R. E., Perforation of the palate and destruction of the nasal bones due to nocardiosis in a Bennett's Wallaby ( <i>Macropus rufogrisca fruticus</i> ) . . . . .	155

## No. 2—April

CAMPBELL, A. COLIN P., The incidence of pathogenic staphylococci in the throat, with special reference to glandular fever . . . . .	157
COOK, G. T., Urease and other biochemical reactions of the Proteus group . . . . .	171
ELEK, STEPHEN D., Rapid identification of Proteus . . . . .	183
WATERWORTH, G. E., and T. H. PULLAR, Adamantinoma of the jaw with pulmonary metastases (plates XXX and XXXI) . . . . .	193
ISAACS, ALICK, Anti-haemolytic action of <i>viridans</i> ( $\alpha$ ) variants of haemolytic streptococci . . . . .	199
COLLINS, DOUGLAS H., Bullet embolism: a case of pulmonary embolism following the entry of a bullet into the right ventricle of the heart (plate XXXII) . . . . .	205
HUMPHREY, J. H., The pathogenesis of glomerulo-nephritis: a re-investigation of the auto-immunisation hypothesis . . . . .	211
PATTISON, I. H., Immunological studies with group-B streptococci . . . . .	219

CAMERON, G. R., G. H. COORAY and S. N. DE, The effect of castration on the action of some barbiturates . . . . .	239
HARTER, ELIZABETH M., and NANCY S. CONWAY, Clotting of human citrated plasma by Gram-negative organisms . . . . .	247
SOLTYS, M. A., Anthrax in a laboratory worker, with observations on the possible source of infection . . . . .	253
PRICE, D. M., The lining of healed but persistent abscess cavities in the lung with epithelium of the ciliated columnar type (plates XXXIII-XXXVI) . . . . .	259
DUGUID, J. P., The influence of cultural conditions on the morphology of <i>Bacterium aerogenes</i> with reference to nuclear bodies and capsule size (plates XXXVII-XXXIX) . . . . .	265
BOLTJES, T. Y. KINGMA, Function and arrangement of bacterial flagella (plates XL-XLVII) . . . . .	275
HARRISON, C. V., Experimental pulmonary arterio-sclerosis (plates XLVIII-LI) . . . . .	289
LENNON, BERNARD, The large-cell small-acinar thyroid tumour of Lainghans and the incidence of related cell groups in the human thyroid (plates LII-LV) . . . . .	295
ENSLIE-SMITH, A. H., Agglutination of coliform bacilli by normal rabbit serum . . . . .	307
DISCOMBE, GEORGE, The quantitative description of the fragility of the erythrocyte and its application to the study of acholuric jaundice . . . . .	315
ŠÍEL, H., Addison's disease due to congenital hypoplasia of the adrenals in an infant aged 33 days (plates LVI and LVII) . . . . .	323
VALLANCE-OWEN, J., The histological demonstration of glycogen in necropsy material . . . . .	325
CRICKSHANK, J. C., A simple method for testing dye sensitivity of <i>Brucella</i> species (plate LVIII) . . . . .	328
INGLIS, ALEXANDER, Squamous metaplasia in the prostate as a result of stilboestrol therapy (plates LIX and LX) . . . . .	330
McFADZEAN, A. J. S., Marrow biopsy in laboratory animals . . . . .	332
HAWKSLEY, J. C., and G. H. COORAY, Observations on fragments of gastric mucous membrane found in aspirated resting gastric juice (plates LXI-LXIII) . . . . .	333
BROWNING, C. H., K. M. CALVER and H. ADAMSON, The chemotherapy of <i>Trypanosoma congolense</i> infection with phenanthridinium compounds, etc. . . . .	336
PERRY, WALTER L. M., The cultivation of <i>Treponema pallidum</i> in tissue culture (plate LXIV) . . . . .	339

	PAGE
WRIGHT, HELEN PAYLING, and DENISE G. EDMONDS, Siderocyte counts in the blood of normal and premature infants . . . . .	342
ADAMSON, W. W., A case of primary sarcoma of the heart (plates LXV and LXVI) . . . . .	344
BOOKS RECEIVED . . . . .	347
PROCEEDINGS OF THE SOCIETY . . . . .	353
MORISON, J. EDGAR, Neoprene latex casts in the study of lung development . . . . .	354

## No. 3—July

BYROM, F. B., and L. F. DODSON, The causation of acute arterial necrosis in hypertensive disease (plate LXVII) . . . . .	357
GOLDIE, W., M. GORDON and K. I. JOHNSTONE, Mutation in a single-cell culture of <i>Corynebacterium diphtheriæ</i> (plate LXVIII) . . . . .	369
CUNNINGHAM, GEORGE J., Trilocular heart with bilateral aneurysmal dilatation of the pulmonary artery (plate LXIX) . . . . .	379
WALTON, KENNETH, and A. G. SPENCER, Ebstein's anomaly of the tricuspid valve (plates LXX and LXXI) . . . . .	387
BRAY, JOHN, and T. E. D. BEAVAN, Slide agglutination of <i>Bacterium coli</i> var. <i>neapolitanum</i> in summer diarrhoea . . . . .	395
JEBB, W. H. H., Starch-fermenting gelatin-liquefying corynebacteria isolated from the human nose and throat . . . . .	403
MOLE, R. H., Fibrinolysin and the fluidity of the blood <i>post mortem</i> . . . . .	413
STRAUB, M., and J. MULDER, Epithelial lesions in the respiratory tract in human influenzal pneumonia (plates LXXII-LXXIV) . . . . .	429
KING, E. J., C. V. HARRISON and G. NAGELSCHMIDT, The effects of kaolin on the lungs of rats (plates LXXV-LXXVIII) . . . . .	435
BARAKAN, T. H., G. R. BARKER, J. M. GULLAND and L. DOROTHY PARSONS, Tissue changes in mice treated with pentose nucleotides and related compounds (plate LXXIX) . . . . .	441
ORR, J. W., and D. E. PRICE, Observations on the hepatotoxic action of the carcinogen <i>p</i> -dimethylaminoazobenzene (plates LXXX-LXXXIII) . . . . .	461
LAUGHTON, NANCY, Canine beta hæmolytic streptococci . . . . .	471
RAEBURN, C., Idiopathic ("isolated") myocarditis in infancy (plate LXXXIV) . . . . .	477

# CONTENTS

xi

	PAGE
WRIGHT, C. J. D., Liposarcoma arising in a simple lipoma (plates LXXXV-LXXXVII) . . . . .	483
SMITH, J. F., Disseminated focal necrosis with eosinophilia and arteritis in a case of asthma (! Löfller's syndrome) (plates LXXXVIII and LXXXIX) . . . . .	489
OAKLEY, C. L., G. HARRIET WARRACK and MARION E. WARREN, The kappa and lambda antigens of <i>Clostridium welchii</i> . . . . .	495
GAULT, E. W., and M. BALASUBRAHMANYAN, A case of cerebral cysticercosis (plate XC) . . . . .	505
SHREWSBURY, J. F. D., and G. J. BARSON, A study of the intestinal flora of <i>Blattella americana</i> . . . . .	506
SHREWSBURY, J. F. D., and G. J. BARSON, A note on the intestinal flora of <i>Melolontha vulgaris</i> . . . . .	510
DOUGHERTY, CARY M., A sebaceous gland in the cervix uteri . . . . .	511
RICKARDS, A. G., and W. MAGAURAN, Fallopian tube granuloma . . . . .	512
MARSHALL, A. H. E., A method for the demonstration of reticulo-endothelial cells in paraffin sections (plate XCI) . . . . .	515
ARMSTRONG, ELIZABETH C., and GEORGIANA M. BONSER, Effect of castration upon the induction of mammary tumours by oestrogen in male mice of the Strong-A strain . . . . .	517
CRUICKSHANK, A. H., Bronchiectasis in laboratory rats . . . . .	520
GORDON, J., A non-gas-producing variant of <i>Bacterium coli</i> induced by growth in glycine . . . . .	521
BOOKS RECEIVED . . . . .	523

## No. 4—October

FENNER, FRANK, The clinical features and pathogenesis of mouse-pox (infectious ectromelia of mice) (plates XCII-XCIV) . . . . .	529
MAITLAND, H. B., and G. MARTYN, A selective medium for isolating <i>Staphylococcus</i> based on the differential inhibiting effect of increased concentrations of sodium chloride . . . . .	553
GINSBERG, A., and A. C. W. LITTLE, Actinomycosis in dogs (plates XCV-XCIX) . . . . .	563
ORR, J. W., D. E. PRICE and L. H. STICKLAND, The glycogen content of rats' livers after poisoning with large doses of <i>p</i> -dimethylaminoazobenzene . . . . .	573
HARRISON, JAMES M., Tuberculosis in a wild sparrow-hawk ( <i>Accipiter nisus nisus</i> ) (plates C and CI) . . . . .	583



	PAGE
JACKSON, A. V., Toxic effects of salicylate on the fœtus and mother . . . . .	587
AMES, C. R., Bacterial variation and toxin production by <i>Corynebacterium diphtheriæ</i> . . . . .	595
SENEVIRATNE, R. D., Redistribution of body fluids in dysenteric toxæmia (plate CII) . . . . .	609
LENNOX, BERNARD, Acute parietal endocarditis in a case of status asthmaticus: a possible early stage of Löffler's endocarditis parietalis fibroplastica with eosinophilia (plates CIII-CVI) . . . .	621
CAMPBELL, A. COLIN P., The bone marrow in glandular fever (plate CVII) . . . .	629
CAMPBELL, A. COLIN P., The histological preparation of bone-marrow particles, uterine curettings and other small tissue fragments . . . . .	633
OBITUARY—	
Edward folliott Creed (plate CVIII) . . . . .	635
David Arthur Welsh (plate CIX) . . . . .	638
BOOKS RECEIVED . . . . .	647
INDEX OF SUBJECTS . . . . .	661
INDEX OF AUTHORS . . . . .	665

# The Journal of Pathology and Bacteriology

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## THE CYTOPLASMIC BASOPHILIA OF MARROW CELLS: THE DISTRIBUTION OF NUCLEIC ACIDS

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(PLATES I-IV)

THE red bone marrow in post-natal human life forms a tissue of great lability. Under normal conditions, by differentiation and mitotic division, it supplies the requirements of the body for new erythrocytes and granulocytes. Under abnormal conditions, particularly in the severe blood dyscrasias, it may undergo considerable hyperplasia, and this is often accompanied by a manifestly heteroplastic hæmopoiesis, fixed and free stem cells accumulating and giving rise frequently to aberrant older forms. This is well seen in the megaloblastic anæmias and in the acute forms of myeloid leukæmia.

In an earlier study (White, 1947), the application of the ribonuclease test of Brachet (1940, 1942) to human sternal marrow suggested that the cytoplasm and nucleoli of the youngest free cells are rich in ribonucleic acid, the amount diminishing progressively with maturation. Few in number in the normal marrow, these are greatly increased in the blood dyscrasias, particularly the severe anæmias. Successful treatment of the primary condition leads to rapid maturation and diminution in number of the primitive cells, with gradual decrease in total cellularity of the marrow and return to normal hæmopoiesis.

The basis of the present work is the attempted substantiation of conclusions drawn from the use of the ribonuclease histochemical test by chemical determination by micro-methods of the nucleic acids in the tissues.

*Intracellular distribution of nucleic acids.* In recent years widespread interest has been focussed upon the distribution of two fundamental types of nucleic acid—the deoxypentose polynucleotides or deoxyribonucleic acids and the pentose polynucleotides or ribonucleic acids—within the cell structures of plant and animal tissues. It is now generally held that the deoxyribonucleic acid type of polynucleotide is confined to nuclear structures—basichromatin of “resting” nuclei and the chromosomes of dividing cells—while the pentose polynucleotides are found in the cytoplasm, mainly in the cytoplasmic granules, but appearing also to enter into the composition of the nucleoli (Davidson, 1946, 1947). The pentose sugar present has been conclusively identified as *d*-ribose only in the case of the polynucleotides from yeast (Gulland and Barker, 1943) and from liver (Davidson and Waymouth, 1944). Nevertheless the term ribonucleic acid is usually applied to pentose polynucleotides in general and will be used in this paper to indicate the pentose polynucleotide from bone marrow, although this material has not yet been fully characterised. /

#### MATERIALS AND METHODS

Samples of red marrow were obtained by sternal puncture biopsy from a series of normal volunteers and individuals suffering from various blood dyscrasias and miscellaneous pathological conditions. In each case 0.1-0.2 ml. of material was aspirated and used for (1) the preparation of films, (2) the preparation of histological sections, (3) chemical analysis.

(1) Six air-dried films were prepared for Romanowsky staining and two films were fixed immediately in the wet state in Susa fluid (Heidenhain, 1916; quoted by Romeis, 1932) for three hours for the ribonuclease test. The Romanowsky-stained films were used for differential cell counts and the construction of maturation curves.

(2) Material was prepared for histology by immediate fixation in 9 to 1 methanol-formol mixture, followed by Susa solution for one hour, centrifuging for ten minutes, and dehydration, post-fixation and double embedding of the tissue by the methods previously described (White, Baker and Griffin, 1946; White, 1947). Sections were cut at  $3\mu$  and stained with iron hæmatoxylin and eosin, Delafield's alum-hæmatoxylin and azur-Giemsa or Leishman's stain mixed with an equal part of veronal-acetate buffer of pH 6.85 for  $\frac{1}{2}$ - $\frac{3}{4}$  hour in the cold, with rapid differentiation in methanol and ethanol, clearing in xylol and mounting in Gurr's neutral mounting medium. These sections reveal the general cellularity and architecture of the marrow.

The ribonuclease test was also performed on sections and wet-fixed films with the following modifications of the method previously given (White).

The purified ribonuclease, prepared by the method of Kunitz (1940-41), was dissolved in distilled water to give a concentration not greater than 0.1 mg. per ml. The solution was adjusted to pH 4.5 by adding, drop-by-drop, *N*/1000 HCl, checking the pH with methyl red on a tile. After heating in a boiling water-bath for three minutes, the solution was cooled and mixed with an equal volume of double-strength veronal-acetate buffer (Michaelis, 1931) of pH 6.85.

The heating at pH 4.5 destroyed traces of contaminating proteolytic enzymes without seriously inactivating the ribonuclease. It is noteworthy, however, that the ribonuclease used had very little adherent proteolytic enzyme activity, since a 0.01 per cent. unheated solution of the enzyme in veronal-acetate buffer removed most of the basophilic material from sections in one hour at 37° C.

without any destructive effect on the general cell outlines; destruction was considerable after 24 hours at 37° C. Although good results were obtained at pH 7.2-7.8 with ribonuclease, it was noticeable that the control tissue in buffer alone also showed reduction in basophilia, although not to the degree produced by the enzyme: there was no action on nuclear chromatin. Using an enzyme solution and control buffer at pH 6.85 (determined by the Beckman glass electrode), it was found that the control tissue in buffer alone showed perfect preservation of cytoplasmic and nucleolar basophilia stainable by pyronin, and of nuclear chromatin stainable by methyl green: the enzyme-treated tissue, on the other hand, showed extensive removal of cytoplasmic and nucleolar basophilia stainable by pyronin, the nuclear chromatin staining with methyl green as in the control.

To carry out the test, the washed films or sections were freed from excess of mercury by iodine followed by sodium thio-sulphate, and washed in tap and distilled water. Paired slides were then treated respectively with (a) the buffer alone at pH 6.85, and (b) enzyme in buffer for one hour at 37° C. The slides were then washed in buffer and stained for one hour in the pyronin-methyl green solution (White). After staining, the slides were washed briefly in water, blotted, taken rapidly through 90 per cent. ethanol and ethanol, cleared in xylol and mounted in Gurr's neutral medium.

The isotonic buffer-enzyme solution had no effect on fresh marrow or blood cells removed straight from the body and suspended in it at 37° C. The basophilic material remained intact after periods of incubation up to several hours. It is possible that the protein nature of ribonuclease rendered intracellular structures inaccessible to its action when separated by the intact cell membrane. Another feature of importance, however, may be the greater stability of the nucleic acid in presence of the enzyme when the acid is bound to native protein. Thorough denaturation of the protein moiety of nucleoproteins, such as occurs in the course of fixation of the marrow cells by Susa fixative, may weaken the protein-nucleic acid bond and partly account for the ready action of the ribonuclease on the subsequent histological preparations. The action of the enzyme is also interfered with in blood or marrow films which have been allowed to dry in the air prior to fixation, and here the protective action of a protein film may operate (see Danielli, 1946). Further work is required for the elucidation of these points.

✓ (3) The third portion of marrow was used for the chemical determination of ribo- and deoxyribonucleic acids. The principle of the method employed is that described by Schmidt and Thannhauser (1945) in which the tissue is extracted with trichloroacetic acid to remove acid-soluble phosphorus and then with lipid solvents to remove lipid phosphorus, and the residue, containing nucleic acid and protein, incubated with alkali which hydrolyses ribonucleic acid to acid-soluble nucleotides but does not affect deoxyribonucleic acid. When the alkaline digest is acidified deoxyribonucleic acid appears in the precipitate and ribonucleic acid remains as acid-soluble nucleotides in the supernatant. /

About half the available specimen was used in procedures (1) and (2) and the remainder was delivered at once into a small weighed tube, 7.5 × 1 cm. Most of the cellular marrow tissue tended to cling to the sides of the tube, the adherent blood running to the bottom. The blood was removed as completely as possible by a capillary pipette and test and the tube stoppered and re-weighed. The tube was then filled with 10 per cent. trichloroacetic acid. After standing for an hour the tissue was freed from the sides of the tube by a glass rod and the tube centrifuged. The supernatant fluid was discarded and the precipitate washed with two successive 5-ml. portions of 10 per cent. trichloroacetic acid. It was then extracted successively with 5-ml. portions of 80 per cent. ethanol, ethanol, and ethanol-chloroform 3 to 1 mixture (two successive extractions for

thirty minutes each at 60°, and finally with ether. The dry residue was dissolved in 1 ml. *N* NaOH (silica free, stored in a waxed bottle), and the digest incubated for 17 hours at 37° C. 1.5 ml. of water were then added, followed by 0.4 ml. of 2.5 *N* HCl and 1.0 ml. of 30 per cent. trichloroacetic acid. The precipitate was centrifuged down and washed twice with 0.5 ml. portions of 5 per cent. trichloroacetic acid, the supernatant fluid and washings being combined.

The precipitate was then ashed with 0.2 ml. of 60 per cent. perchloric acid and the phosphorus determined by the method of Berenblum and Chain (1938) as modified by Davidson and Waymouth (1943). This gave the deoxyribonucleic acid phosphorus (DNAP). The supernatant fluid and washings were made up to 5 ml., and 2 ml. were pipetted into a short wide pyrex test-tube and taken to dryness on the water-bath. The residue was then ashed with 0.2 ml. of perchloric acid and the phosphorus determined as above. This gave the ribonucleic acid phosphorus (RNAP).

With each set of estimations a reagent blank was run through in order to allow for impurities in the reagents employed. The final colours were read off in the Hilger Spekker absorbtimeter.

## RESULTS

### NORMAL SERIES

The subjects were 15 healthy volunteers of both sexes, ranging in age from 17 to 43 years. Normal blood counts and reticulocyte levels were found in all.

#### *The general architecture of the bone marrow*

Representative sections from 4 individuals are shown in figs. 1-4 stained by various methods. The variability in the normal "lattice" pattern of large fat cells and cellular centres of hæmopoiesis is seen, and in all 15 subjects the pattern was repeated within these limits. In the hæmopoietic centres it can be seen that primitive free cells with basophilic cytoplasm and nucleoli are few in numbers. Partly differentiated forms—erythroblasts and myelocytes—are more numerous, and older cells—normoblasts and band-form and segmented granulocytes—are still more abundant. Where the section cuts across marrow sinusoids, the developing cells are seen to be always in the stroma, lying extravascularly (figs. 3 and 27). Intensely basophilic cytoplasm, due to the presence of ribonucleic acid and removable by the action of ribonuclease, is found in the plasma cells as well as in the youngest hæmopoietic cells. The cytoplasmic basophilia diminishes very rapidly with maturation of the older erythroblasts and granulocytic cells, particularly the latter.

#### *The differential cell counts and maturation curves from marrow films*

The system of classification is based on a unitarian view of hæmopoiesis, similar to that of Downey (1924, 1938), Ferrata and Fieschi (1940) and Rastelli (1943) and following the views of Maximow

## NORMAL HUMAN SPLEEN MARROW

Sections to show normal structure and range of cellularity

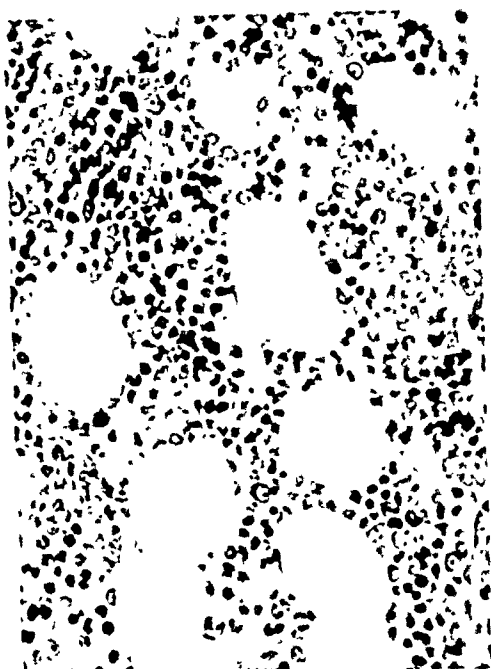


FIG. 1.—Male, M. W., aged 26. Delafield's alum-haematoxylin and azur II—Giemsa.

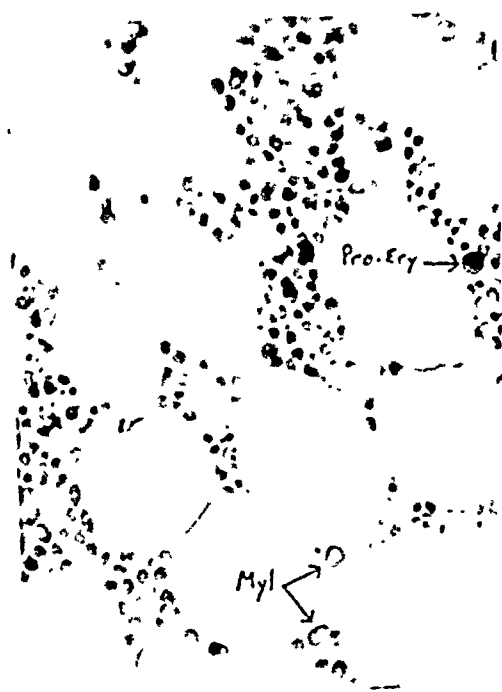


FIG. 2.—Male, D. J., aged 43. Pyronin-methyl green. Pro. Ery. pro-erythroblast. Myl. myelocytes.



FIG. 3.—Male, B. M., aged 22. Iron-haematoxylin and eosin. Sin. = sinusoid.

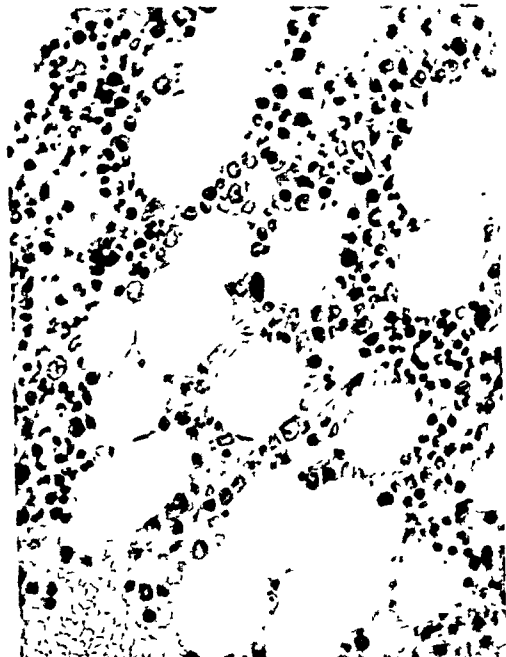


FIG. 4.—Male, B. B., aged 23. Iron-haematoxylin and eosin.

All sections  $3\mu$ , methyl alcohol-formol and Susa-fixed, and  $\times 300$ .



TABLE I  
Cytological characteristics at different stages of red cell and granulocyte development

Cell type	✓ Cytoplasm : basophilia (ribonucleic acid content)		Granulation	✓ Nucleolus : basophilia (azurophilic granules, etc. (ribonucleic acid content))	Nucleolus associated chromatin detectable by Feulgen reaction
	Erythropoiesis	Granulopoiesis			
Fixed stem cell or hemoblast (Ferrata), derived from mesenchymal remnant of Maximow	Slight to +		Nil or a few fine azurophilic granules	Slight to +	Slight
Free stem cell or hemoblast (myeloblast of Downey)	+++		Nil	++	Slight
Erythropoiesis	Erythropoiesis	Granulopoiesis	Erythropoiesis	Erythropoiesis	Erythropoiesis
Pro-erythroblast	++++	++	Nil	++	Very slight to +
Basophilic erythroblast	++++	++	Nil	Very slight to nil	Very slight to +
Polychromatic erythroblast	+++ to ++	+	Nil	Nil	Not distinguishable from nucleolar chromatin
Meta-myelocyte (3 types)*	Very slight	Very slight to nil	Nil	Nil	Remnants visible in nucleolar chromatin
Band-form granulocyte (3 types)*	Very slight to nil	Nil	Nil	Nil	Nucleolar pyknotic
Segmented granulocyte (3 types)*	...	...	...	...	...

\* Neutrophil, eosinophil, basophil.

† Slight diffuse cytoplasmic basophilia revealed by ordinary methods, at least in the Feulgen reaction.



(1927) on the potentialities of the primitive mesenchymal remnant associated with the reticulum of the marrow stroma, but differing from his views on the equivalence of the haemocyto blast (myeloblast of Downey) to the small lymphocyte, which he considered to function as a pluripotent cell, at least under certain conditions. Most of the lymphocytes in normal and indeed in pathological marrow smears are

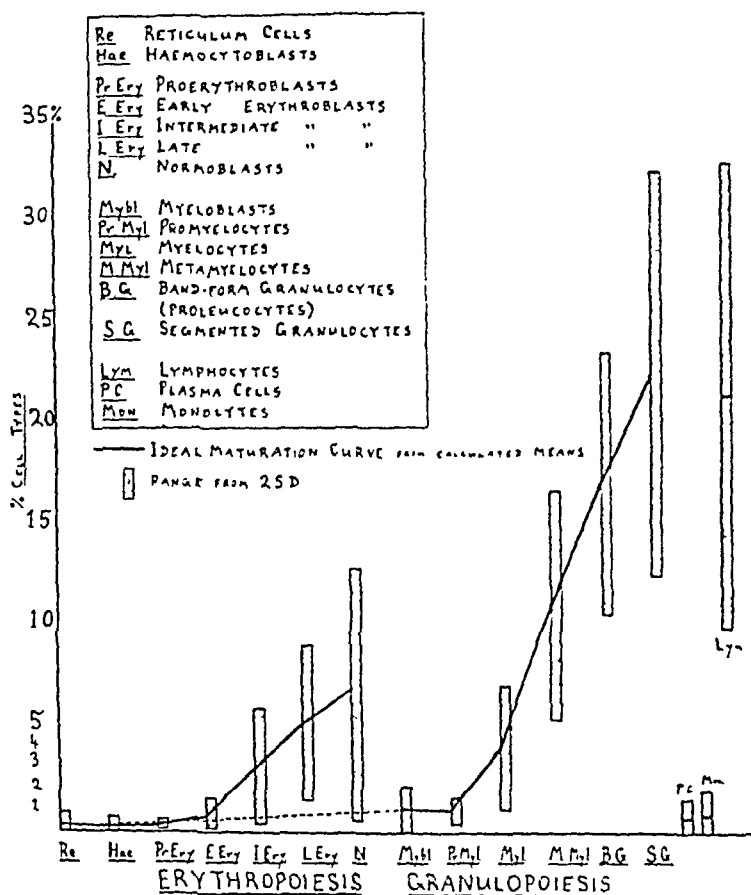


FIG. 5.—Maturation curves for normal human marrow cells based on sternal-puncture examination of 15 subjects. ( $6 \times 1000$ ,  $9 \times 500$  cell counts.)

in number and this part of each curve inclines only very slightly. The steepness increases rapidly with increasing maturity, and in both series the stages at which mitotic divisions are most frequently encountered show rapid numerical increase.

In general, mitoses in normal marrow are more frequently seen in the red-cell than in the granulocytic series, despite the numerical superiority of the latter as shown by the ratio of total granulocytes to nucleated red-cell stages. When the leuco-erythrogenetic ratio (Pontoni, 1936) is considered, taking the ratio of young granulocytes from myeloblast to metamyelocyte stages and the total nucleated red cells, the disparity in the two series is less marked (normal observed range 0.56-2.67). In the red cell series, mitoses are most frequently encountered in the basophilic and polychromatic erythroblasts, and in the granulocytic series at the myelocyte stage. Normally, stem-cells in mitosis are encountered very rarely.

The wide range of mature granulocytes and of lymphocytes may be partly explained by the admixture of the aspirated marrow with varying amounts of blood with its own leucocyte population.

#### *The distribution of ribo- and deoxyribonucleic acids*

The results of analysis of 10 normal specimens, with calculated parameters, are shown in table II. Like the cellular population of

TABLE II

*Nucleic acid content of normal human sternal bone marrow*  
*Values for 10 individuals, expressed as mg. of phosphorus (P)*  
*per 100 g. of fresh tissue*

Subject, sex, age	Wt. of marrow sample (mg.)	Total nucleic acid P (TNAP)	Ribonucleic acid P (RNAP)	Deoxyribonucleic acid P (DNAP)
1. D. L. ♀ 17	31.0	29.0	18.3	10.7
2. B. M. ♂ 22	39.5	28.2	16.4	11.8
3. D. S. ♂ 20	57.5	26.5	13.5	13.0
4. S. J. ♀ 20	44.0	23.6	17.2	6.4
5. B. B. ♂ 23	22.6	22.2	15.2	7.0
6. M. W. ♂ 26	60.0	18.4	12.2	6.2
7. D. J.* ♂ 43	36.0	16.1	12.2	3.9
8. P. P. ♂ 19	15.6	15.6	11.7	3.9
9. E. J.* ♂ 40	59.0	14.3	10.5	3.8
10. W. L. ♂ 35	35.0	13.0	10.3	2.7
<i>True means</i>		20.7	14.2	6.9
<i>Standard deviation (S.D.)</i>		5.0	3.6	3.5
<i>Range (mean ± S.D.)</i>		32.5 to 8.9	21.4 to 7.0	13.9 to Zero

\* Brothers.

the marrow, the results show fairly wide variations, which is hardly surprising in such a heterogeneous tissue, liable to be contaminated to a varying extent with peripheral blood.

## ABNORMAL SERIES

The results for the abnormal series are set out in table III, together with relevant information on the condition of the peripheral blood. In a number of cases the examination was repeated at intervals during the course of treatment. The results will be considered in groups on the basis of aetiology.

## The anæmias

In conditions with clinical anæmia and depressed erythrocyte and hæmoglobin levels in the peripheral blood, a hyperplastic marrow is frequently found, with elevated nucleic-acid values. This is particularly marked where the maturation of the red-cell series is altered, with accumulation of the more primitive forms. In cases followed throughout the course of response to specific therapy, a fall in nucleic-acid levels to within normal limits is found to accompany the return to a normal marrow histology and peripheral blood picture.

*Pernicious anæmia*

In this group, 5 patients (cases 1-5 in table III) were examined in the untreated state, 4 of these being re-examined at intervals during the course of response to specific therapy. Four of the 5 cases showed, in the untreated state, clinical and morphological anæmia of different degrees of severity, accompanied by a hyperplastic megaloblastic sternal bone marrow and elevated marrow nucleic-acid levels. The fifth case (no. 5) showed moderate macrocytic anæmia with slightly hypoplastic but megaloblastic marrow, and low nucleic-acid levels. The diagnosis in all cases was substantiated by the finding of histamine-fast gastric achlorhydria, glossitis, elevated serum bilirubin and varying degrees of peripheral and central nervous system involvement, in addition to macrocytic anæmia with megaloblastic marrow.

**Case 1.** A. H., female, aged 66. Moderately severe pernicious anæmia with some peripheral and C.N.S. involvement and an acute urinary tract infection. The infection responded quickly to sulphadiazine, and simultaneously administered oral folic acid (20 mg.  $\times$  2 days, 5 mg.  $\times$  6 days) produced a reticulocyte response of 48 per cent. on the 7th day. Rapid improvement in the peripheral blood occurred during and after the reticulocyte response, and, after 26 days, therapy was continued with purified liver extract parenterally (Examen 2 ml.).

Before therapy, the sternal marrow showed considerable hyperplasia, with few fat cells, prominence of basophilic hæmocyto blasts and pro-erythroblasts, and characteristic megaloblastic erythropoiesis with greatly reduced normal-type erythropoiesis (fig. 6). The young megaloblasts with slight or no hæmoglobinisation possessed abundant basophilic material in their cytoplasm. The granulocyte series showed a number of giant bizarre forms of metamyelocytes and band forms (giant stabs). The nucleic-acid values showed an elevation of ribonucleic-acid phosphorus (RNAP) to 41.4 mg./100 g., more than 3 S.D. above the mean of the normal series. The deoxyribonucleic-acid phosphorus



## PLATE II

### PERNICIOUS ANÆMIA IN RELAPSE. Case 2. (C. B.)

*The sternal marrow structure before and during the course of liver therapy*

FIG. 12.—Before therapy. Hyperplasia; megaloblastic erythropoiesis. Pyronin-methyl green after pH 6.85 veronal-acetate buffer for 1 hr. at 37° C. Hæ. = hæmocyto blasts. Meg. = groups of megaloblasts.

FIG. 13.—Similar section stained by pyronin-methyl green after ribonuclease treatment for 1 hr. at pH 6.85 and 37° C. Note removal of basophilia from cytoplasm and nucleoli of young cells.

FIG. 14.—At reticulocyte peak. Similar cellularity to fig. 12, but normoblastic erythropoiesis. Pyronin-methyl green.

FIG. 15.—During recovery. Less hyperplastic, with more normal hæmopoiesis. Pyronin-methyl green after buffer.

FIG. 16.—Similar section stained by pyronin-methyl green after ribonuclease. Cells showing considerable depletion of basophilia are now less numerous.

FIG. 17.—On recovery. Cellularity and hæmopoiesis are now normal. Pyronin-methyl green.

All sections 3  $\mu$ , methyl alcohol-formol and Susa-fixed,  $\times 300$ .

STRUCTURAL ANALYSIS OF THE CLATHRATE A. IX

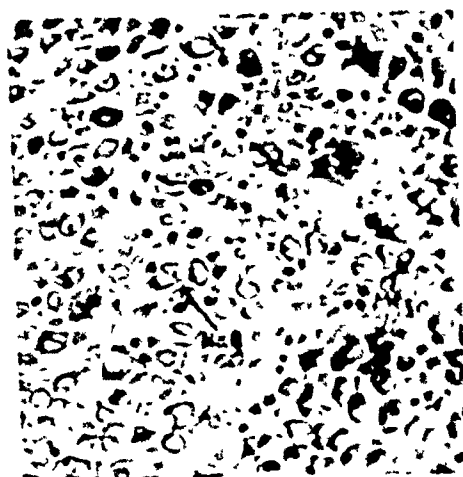


Fig. 12.

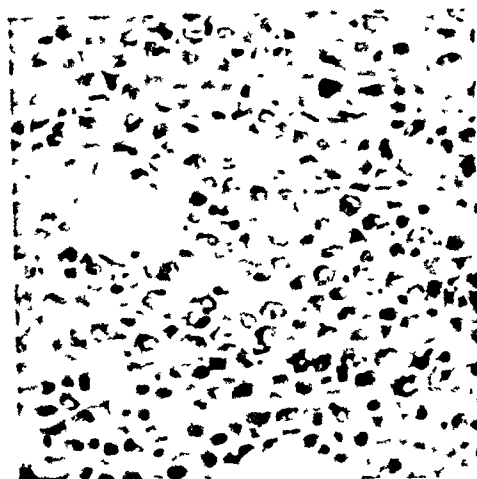


Fig. 13.

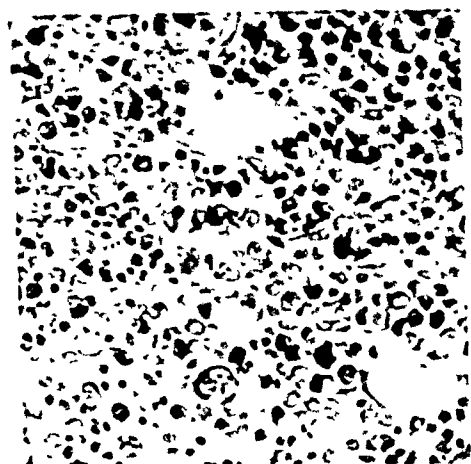


Fig. 14.

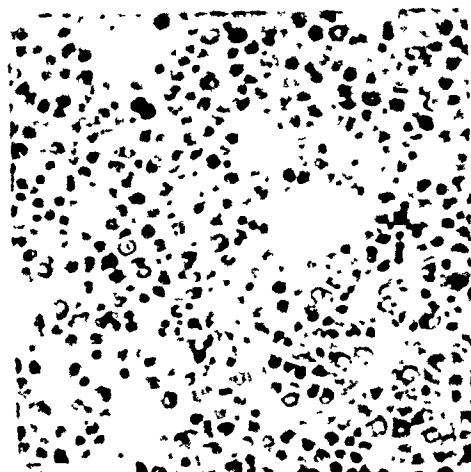


Fig. 15.

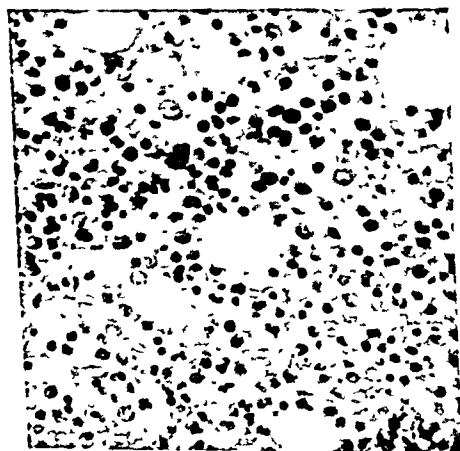


Fig. 16.

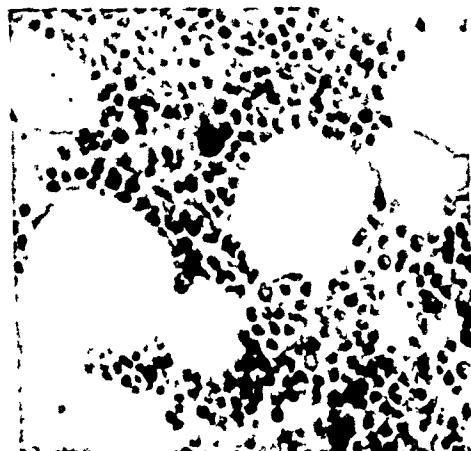


Fig. 17.







### PLATE III

- FIG. 18.—Pernicious anemia in relapse. Case 5 (M. C.). There is relative hypocellularity in spite of fairly numerous early cells and some degree of megaloblastic erythropoiesis. Pyronin-methyl green.  $\times 300$ .
- FIG. 19.—Familial hæmolytic anemia. Case 14 (M. D.). Considerable hyperplasia, with predominance of pro-erythroblasts, erythroblasts and normoblasts. Pyronin-methyl green after veronal-acetate buffer pH 6.85 for 1 hr. at 37° C.  $\times 300$ .
- FIG. 20.—Similar section,  $\times 580$ . Pro. Ery. = pro-erythroblasts. I. Ery. = intermediate (polychromatic) erythroblasts. N. = normoblasts.
- FIG. 21.—Similar section,  $\times 580$ . Pyronin-methyl green staining after ribonuclease action for 1 hr. at pH 6.85 and 37° C. Note removal of basophilia from cytoplasm and nucleoli of young cells. Nuclear chromatin and nucleolus-associated chromatin unaffected.

STERNAL MARROW: THROMBOCYTOSIS IN MYELOID LEUKAEMIA AND ESSENTIAL  
THROMBOCYTHAEMIA (1921)

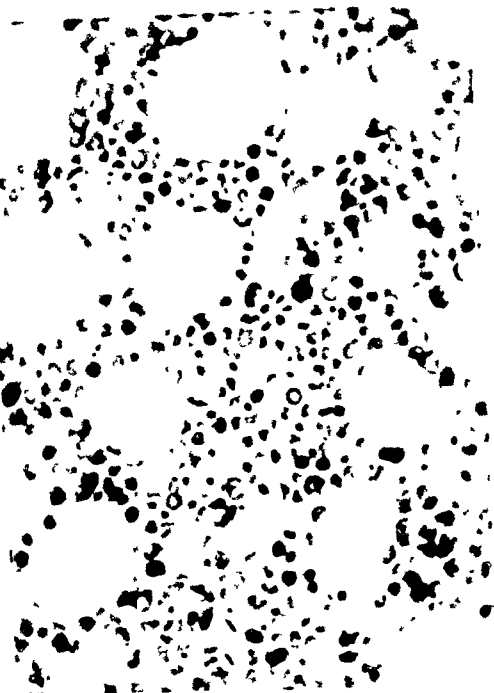


FIG. 18.

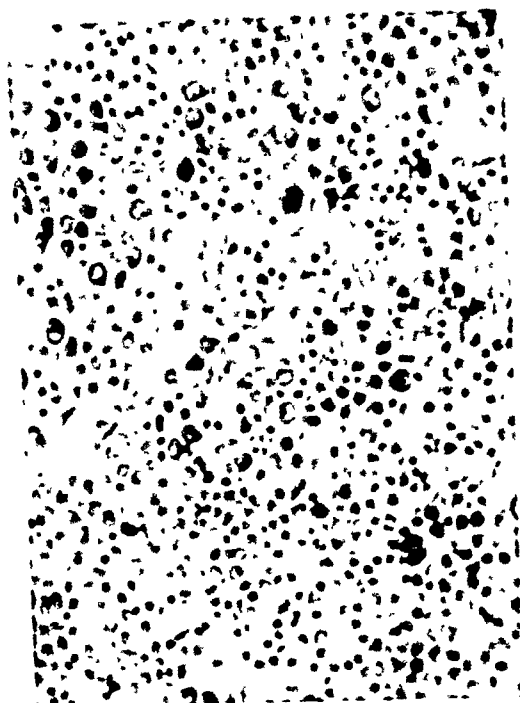


FIG. 19.



FIG. 20.

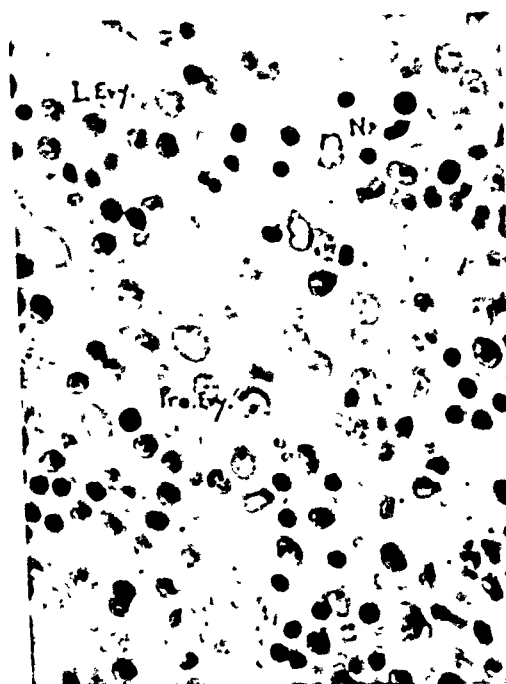


FIG. 21.



(DNAP) was elevated to 30.6 mg./100 g., the increase also exceeding 3 S.D. above the mean of the normal series (table III).

Five days after commencing specific therapy, when the reticulocytes were 20 per cent., the sternal marrow showed greatly accelerated erythropoiesis, with predominance of late erythroblasts and normoblasts. Fixed and free stem-cells were now reduced to more normal numbers, the megaloblastic series was represented only by a few obvious forms, and occasional intermediate forms. In the differential count, the mature granulocytes and lymphocytes were relatively reduced. The total cellularity, as judged from the sections, did not appear to differ greatly from that before treatment. The nucleic acid levels were considerably reduced, particularly the RNAP, which now lay just above the upper normal range, though the DNAP was still considerably elevated.

Further sternal punctures were performed near the end of the reticulocyte response (19 days from starting treatment), and 26 days from the onset, when the blood count was  $4 \times 10^6$  and the Hb. 11.7 g. There showed a return of the maturation curves for erythropoiesis to within the normal range, with more gradual reduction in cellularity and a return to the normal architecture of the marrow. These changes were accompanied by a further fall in the nucleic acids to within the normal range (figs. 7-10 and table III).

**Case 2. C. B., female, aged 74.** This old-standing case in severe relapse (10 months lapse of treatment) was given purified liver extract (Anolaxmin) parenterally and ferrous sulphate orally. The findings before and during therapy closely resemble those in the previous case. The reticulocyte peak occurred on the 11th day, and on the 12th day the sternal marrow showed reduction in stem-cells and pro-erythroblasts from a considerably augmented initial level to within normal limits. There was a predominance of late erythroblasts and particularly of orthochromatic normoblasts, with absence of the megaloblastic series and only a few intermediate types. The total cellularity was similar to that existing before therapy. The nucleic acids were reduced from an augmented level—above the means for the normal series of more than 3 S.D. for the RNAP and 2 S.D. for DNAP—to within normal limits (table III). The reduction in DNAP was particularly striking and may well be correlated with the excess of orthochromatic normoblasts with small pyknotic nuclei present at the time.

Later, with the return of the maturation curves to within normal limits and a gradual reduction in cellularity and return to a normal architecture, the values for nucleic acids fitted more closely into the mid-normal ranges, RNAP falling and DNAP rising somewhat. These changes are shown in table III and in figs. 11-17.

Results from the other three patients in this group (cases 3-5) are shown in fig. 18 (case 5) and table III.

### *Refractory megaloblastic anæmias*

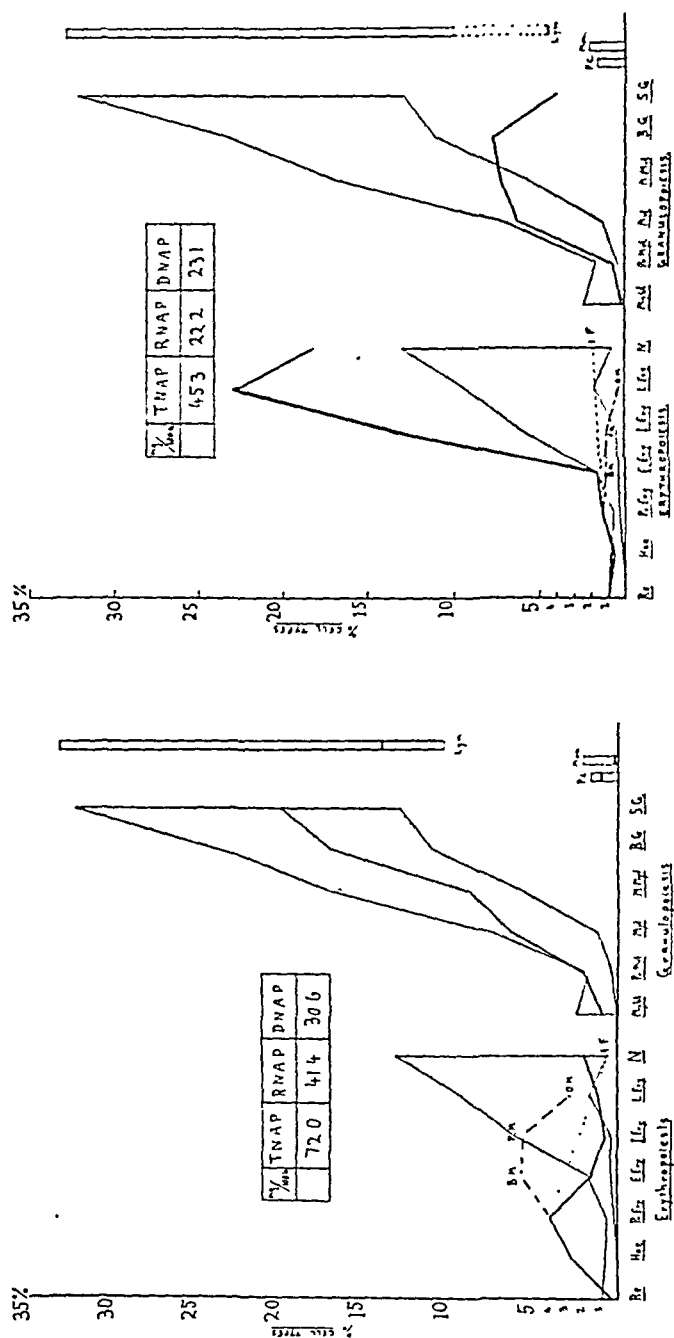
Two patients, E. B. and G. E. (cases 6 and 7), treated with proteolysed liver are shown in table III.

### *Macrocytic anæmias of other ætiology*

**Case 8. C. M., male, aged 37,** whilst on service in the Middle East, had developed a macrocytic anæmia with sprue-syndrome glossitis ("raw beef" tongue), angular stomatitis, achlorhydria and intermittent fatty diarrhoea, his fat absorption ranging from 65 to 90 per cent. on a daily fat intake of 50 g. Response to liver therapy had been variable, and the present investigation, 2 years from the onset, showed a moderate macrocytic anæmia with a fairly

hyperplastic marrow rich in stem cells, pro-erythroblasts and the earlier erythroblasts, but poor in older nucleated red cells (figs. 22-25). Megaloblastic

MATURATION CURVES OF STERNAL MARROW CELLS IN PERNICIOUS ANEMIA BEFORE AND DURING TREATMENT



erythropoiesis was absent, but the cells were often large at even advanced stages of hæmoglobinisation, with rather open nuclear chromatin, and erythro-



TABLE III—Summary of results in the abnormal series

Case no.	Diagnosis	Peripheral blood picture					Structural features of sternal marrow	Nucleic acid distribution in sternal marrow expressed as mg. P/100 g. of tissue.		
		R.B.C. ( $\times 10^6$ per c.mm.)	Hb. (g. per 100 c.c.)	C.I.	Leuco- cytes per c.mm.	Reticulo- cytes (per cent.)		TNAP	RNAP	DNAP
1. A. H.	Pernicious anaemia; before therapy	1.3	6.5	1.58	6000	2.4	Hyperplastic; megaloblastic erythropoiesis	72.0	41.4	30.6
	Shortly before reticulocyte peak	1.4	6.5	...	...	28.8	Hyperplastic; normoblastic erythropoiesis	45.3	22.2	23.1
	Near end of reticulocyte peak	...	9.4	...	...	10.0	Decreasing cellularity; normal hemopoiesis	33.3	20.1	13.2
	During recovery	4.0	11.7	0.93	7000	2.0	Normal cellularity and hemopoiesis	10.7	8.5	2.2
2. C. B.	Pernicious anaemia in relapse; before therapy	1.7	6.7	1.26	3000	1.8	Hyperplastic; megaloblastic erythropoiesis	52.0	37.0	15.0
	At reticulocyte peak	1.6	5.8	...	...	33.0	Hyperplastic; normoblastic erythropoiesis	25.1	21.5	3.6
	During recovery	4.6	12.3	...	6000	1.7	Decreasing cellularity; normal hemopoiesis	28.4	20.0	8.4
3. C. G.	At recovery, with restoration of normal blood picture	5.4	15.25	0.85	6000	0.6	Normal cellularity and hemopoiesis	24.3	13.8	10.5
	Pernicious anaemia in relapse; before therapy	1.0	9.0	1.48	8000	0.6	Hyperplastic; megaloblastic erythropoiesis	42.2	25.4	16.8
	Early in reticulocyte response	2.0	7.5	1.2	...	14.8	Hyperplastic; normoblastic erythropoiesis	7.1	4.9	2.2
4. F. K.	Towards end of reticulocyte response	2.7	9.7	1.16	5000	3.3	Decreasing cellularity; normal hemopoiesis	14.5	9.1	5.4
	Pernicious anaemia; before therapy	...	...	...	...	...	Hyperplastic; megaloblastic erythropoiesis	44.1	27.1	16.9
	Pernicious anaemia; before therapy	2.5	11.4	1.4	5000	2.2	Slightly hypoplastic; megaloblastic erythropoiesis	10.3	6.4	3.9
5. M. C.	During reticulocyte response	3.0	13.3	1.41	7000	12.4	Slightly hypoplastic; normoblastic erythropoiesis	20.2	12.9	7.3
	Refractory megaloblastic anaemia; during therapy	1.0	7.3	1.24	1000	2.4	Hyperplastic; megaloblastic type of erythropoiesis with many hemohistioblasts	37.3	23.5	13.8
6. E. B.	Refractory megaloblastic anaemia; slight relapse after 3 yrs. remission	3.3	13.5	1.3	2000	1.4	Slightly hyperplastic; normal type of erythropoiesis with prominence of erythroblasts	38.0	21.0	17.0
7. G. E.	Macrocytic anaemia (sprue syndrome); before therapy	3.2	13.0	1.3	4900	1.5	Moderately hyperplastic; intermediate type of erythropoiesis	42.0	25.0	15.2
	Following parenteral purified liver extract; 6 days from 1st injection of Anahamin	4.3	14.2	1.05	6000	4.9	Moderately hyperplastic; normal type of erythropoiesis	36.1	23.8	12.3
	30 days from onset of therapy	4.6	15.9	1.1	7000	1.1	Normal cellularity and hemopoiesis	23.5	15.5	8.0

9. F. H.	Macrocytic anemia (former tuberculous peritonitis); before therapy	2.3	8.74	1.5	5000	2.2	Hyperplastic; largely intermediate type of erythropoiesis		
	Early in reticulocyte response.	1.8	9.0	1.5	...	0.2	Hyperplastic; more normal erythropoiesis		
	At reticulocyte peak (12.1 per cent.)	1.8	9.7	1.7	...	12.1	Cellularity slightly reduced; erythropoiesis still partly abnormal		
	1 month from end of 1st course of folic acid, during 2nd course with pyridoxin	2.8	10.0	1.22	...	2.0	Cellularity much reduced; erythropoiesis reduced but still slightly abnormal		
10. K. L.	Nocturnal haemoglobinuria	1.8	7.8	1.4	5000	35.0	Hyperplastic; predominance of fairly mature erythroblasts and of normoblasts		
11. R. L.	Celiac disease; condition inactive	1.8	10.0	0.71	5000	1.3	Normal cellularity and haemopoiesis		
12. A. L.	Celiac disease; condition inactive	1.0	12.0	1.01	5000	1.0	Normal cellularity and haemopoiesis		
13. M. H.	Celiac disease; condition inactive	1.8	10.8	0.72	...	...	Moderately hyperplastic; action normal hemopoiesis		
14. M. D.	Familial hemolytic anemia	2.5	7.8	1.0	9000	33.0	Hyperplastic; very active normal type erythropoiesis, many young erythroblasts		
15. H.	Familial aplastic anemia; in remission after splenectomy	1.9	10.85	...	10,000	<0.1	Normal cellularity and haemopoiesis		
16. A. G.	Chronic hypochromic anemia; early in therapy	1.5	9.7	0.7	12,000	...	Slightly hyperplastic; erythroblasts numerous		
17. M. H.	Sulphhaemoglobinemia; fibrositis	1.5	11.2	...	...	...	Normal cellularity and haemopoiesis		
18. O. F.	Toxic purpura; empyema	1.9	13.6	0.85	9000	...	Normal cellularity and haemopoiesis		
19. H. M.	Polycythemia vera; during therapy by repeated venesection	1.6	11.2	...	15,000	3.2	Moderately hyperplastic; rather many predominant		
20. J. R.	Polycythemia vera; haematemesis	0.6	11.0	0.68	16,000	1.6	Moderately hyperplastic; numerous erythroblasts, myelocytes in frequency of 1 per cent.		
21. E. H.	Chronic myeloid leukemia; before deep X-ray therapy	1.1	13.3	1.03	220,000	...	Hyperplastic; great predominance of erythrocytes and younger granulocytes		
	6 weeks later, after deep X-ray therapy for 1 month	5.0	15.0	0.96	13,000	...	Section not obtained; films obtained in all cell types, particularly primitive granular cells		
22. M. O.	Chronic myeloid leukemia	3.8	11.1	0.93	35,000	...	Hyperplastic; great predominance of myelocytes and younger granulocytes		



Hæmopoietic response was obtained with parenteral purified liver extract (Anahæmin); after 7 days the reticulocytes were 4.9 per cent. and the sternal marrow showed a greater degree of maturity, the red-cell maturation curve falling within the normal range. The nucleic-acid levels now showed a fall of RNAP to just above the upper normal range, whilst the DNAP was within the normal range. Thirty days from commencing treatment, the marrow structure and maturation curves approached more nearly to normal, and the nucleic-acid levels were in the mid-normal ranges.

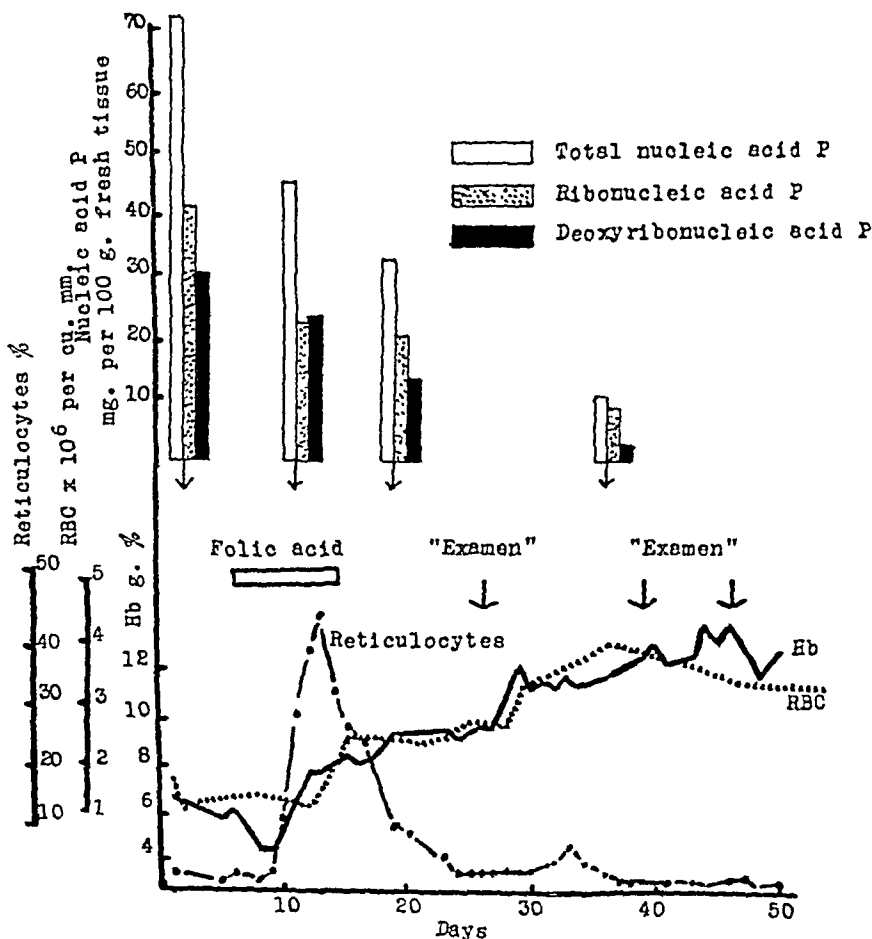


FIG. 10.—Case 1 (A. H.). Pernicious anaemia. Nucleic acid levels in the sternal marrow shown in relation to the peripheral blood picture during response to therapy.

The blood picture gradually became normal, but it is noteworthy that the glossitis and angular stomatitis persisted and were resistant to oral riboflavin 35 mg.  $\times$  5 days and nicotinic acid 10 mg.  $\times$  7, 5 mg.  $\times$  7 days, but improved steadily with bi-weekly injections of 4 ml. of crude liver extract (Plexan), the papillae regenerating and the epithelium healing. The degree of vascularisation of the corneo-scleral junctions also diminished. Plexan also maintained a perfectly normal blood picture.



#### PLATE IV

##### Macrocytic anaemia (sprue syndrome). Case 8. (C. M.)

FIG. 22.—Before therapy. Moderately hyperplastic immature marrow. Erythropoiesis of intermediate type. Hæ. = hæmocyto blasts. Myl. = myelocyte. Pyronin-methyl green after pH 6.85 veronal-acetate buffer for 1 hr. at 37° C.

FIG. 23.—Similar section stained by pyronin-methyl green after action of ribonuclease for 1 hr. at pH 6.85 and 37° C.

FIG. 24.—Marrow after liver therapy (anahæmin). Cellularity does not differ greatly from that before therapy, but greater degree of maturity. Pyronin-methyl green after buffer.

FIG. 25.—Similar section, stained by pyronin-methyl green after ribonuclease action.

##### Macrocytic anaemia. Former tuberculous peritonitis. Case 9. (F. H.)

FIG. 26.—Hyperplastic immature marrow with intermediate type of erythropoiesis. Hæ. = group of hæmocyto blasts. Pyronin-methyl green after buffer.

FIG. 27.—Similar section stained by pyronin-methyl green after ribonuclease action. Hæ. = hæmocyto blasts. Sin. = sinusoid.

FIG. 28.—Marrow early in the course of therapy with folic acid. Cellularity similar to that before therapy, but greater degree of maturity. Cap. = capillary.

All sections 3  $\mu$ , methyl alcohol-formol and Susa fixed,  $\times 300$ .

STERNAL MARROW IN MACROCYTIC ANÆMIA

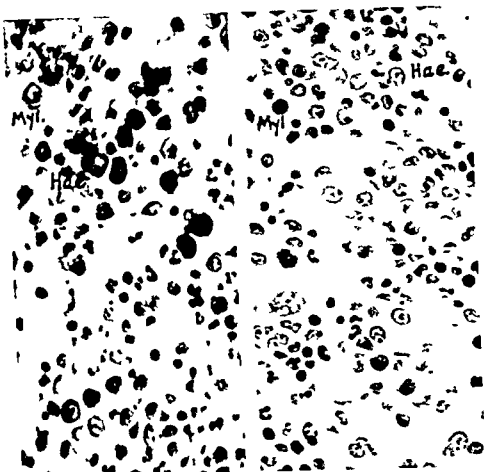


FIG. 22.

FIG. 23.

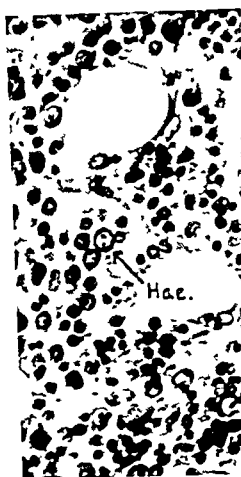


FIG. 24.

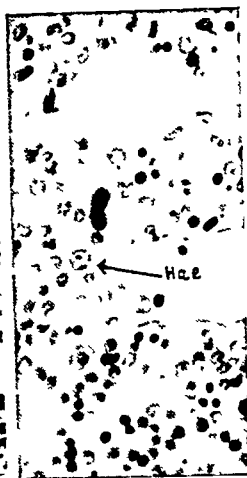


FIG. 25.

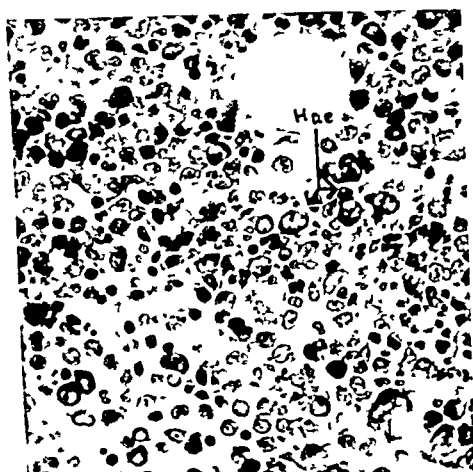


FIG. 26.

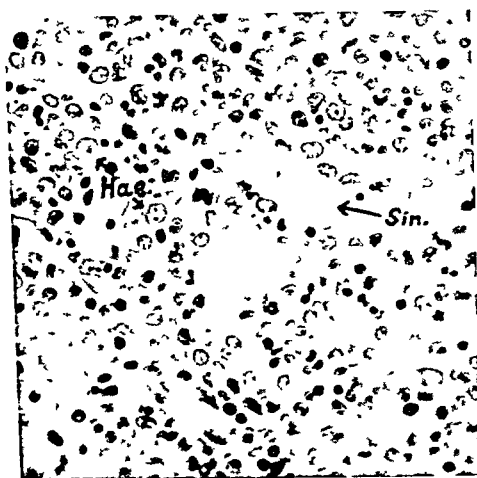


FIG. 27.

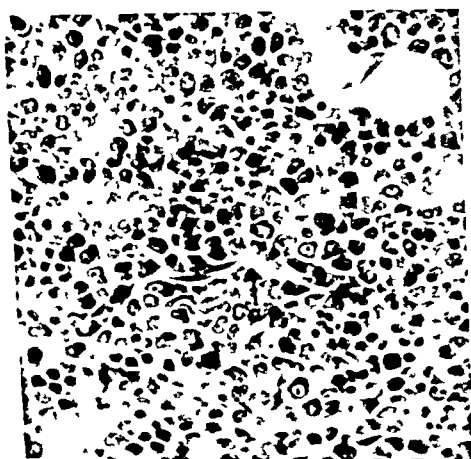


FIG. 28.



Case 9. F. H., female, aged 57. Plastic tuberculous peritonitis at 18 years of age was followed by macrocytic anaemia after 10 years and the development of subacute obstruction after 20 years. Operative intervention and liver-extract therapy enabled the patient to maintain fair health, but with periodic attacks of oedema with hypoproteinaemia and macrocytic anaemia. The present investigation disclosed moderate macrocytic anaemia and slight hypoproteinaemia, with normal plasma bilirubin. The glucose-tolerance curves and faecal-fat analyses were normal. The sternal marrow showed considerable hyperplasia, with some excess of pro-erythroblasts and the earlier erythroblasts. The majority of developing red cells were normal in type, but a small number of typical megaloblasts were present and a larger number of large, haemoglobinising cells intermediate in type between the two series (figs. 26-28).

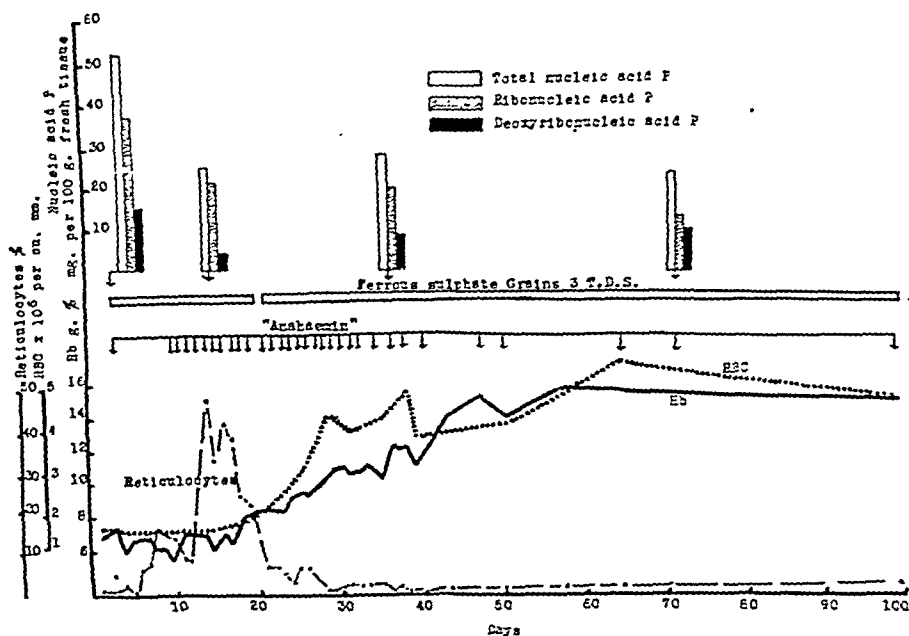


FIG. 11.—Case 2 (C. B.). Pernicious anaemia in relapse. Nucleic acid levels in the sternal marrow shown in relation to the peripheral blood picture during response to therapy.

The nucleic-acid levels were considerably increased, the elevation exceeding 3 S.D. of the normal mean values in each case, and greater in the case of the RNAP—56 mg./100 g. (table III). Treatment by oral folic acid (25 mg.  $\times$  4 days, 10 mg.  $\times$  5 days) produced a reticulocyte response of 12.1 per cent. and a gradual rise in the packed cell volume from 26 to 32. This rather slight improvement in the peripheral blood was accompanied by a change in the marrow structure, which now showed more normal maturation of the red-cell series, with gradual reduction in total cellularity towards normal (figs. 27 and 28). These changes were accompanied by a steady fall in the nucleic-acid levels to within normal limits. However, the peripheral blood picture did not improve further after a second course of folic acid, together with pyridoxin, although slight reticulocytosis continued. Anabæmin (2 ml.  $\times$  3 days) followed by Plexan (2 ml.  $\times$  7 days) was then given, and 5 weeks later the packed cell volume was 40.5, with erythrocytes  $4.0 \times 10^6$  and Hb 13.73 g., C.I. 1.1. Considerable oedema was still present and the plasma protein level was now 4.6 g. per cent.

### Other pathological states

Results from a number of other cases of different types are summarised in table III \*: only one will be considered in detail.

#### *Chronic myeloid leukaemia*

**Case 21.** E. H., female, aged 72. 7 months history. Successful response to deep X-ray therapy 6 months previously, with reappearance of enlarged spleen and lymph nodes and a rise in the blood granulocytes after 4 months. The peripheral blood showed all stages of granulocytic differentiation, with 10 per cent. myeloblasts in a 220,000 leucocyte count. The marrow was hyperplastic, with great predominance of granulocytes, and frequent promyelocytes, myelocytes and metamyelocytes. The nucleic-acid levels showed an elevation of the DNAP to 3 S.D. above the normal mean: the RNAP was within normal limits (table III). Deep X-ray therapy (400 r) over a period of three weeks produced a fall of blood leucocytes to 13,000, with a practically normal differential count. The sternal marrow now showed evidence of greatly reduced cellularity. A section was not obtained, but the films were poor in immature cells of both the red-cell and granulocytic series. The nucleic-acid levels were now in the lower ranges of normal for both forms.

### DISCUSSION

These results support the conclusions drawn earlier from the application of the ribonuclease test to human marrow (White, 1947). The variations from normal in the distribution of ribo- and deoxyribonucleic acids, as shown histochemically and by chemical micro-analysis, are closely related to changes in histological structure.

The findings may be discussed both in relation to problems of hæmopoiesis and to the question of actively growing and embryonal tissues generally. Evidence has accumulated that cells which proliferate rapidly or synthesise much cytoplasmic protein are often rich in cytoplasmic and nucleolar concentrations of ribonucleic acid (Caspersson and Schultz, 1939, 1940; Caspersson and Santesson, 1942). Brachet (1942, 1945) has made similar observations on embryonic and growing tissues, and points out that ribonucleic acid may be largely localised in the mitochondria and small cytoplasmic particles (microsomes; Claude, 1943 *a* and *b*), and considers these structures to be centres for protein synthesis.

In normal marrow the distribution of nucleic acids varies widely, but this tissue has a variable normal constitution, as is evident from the histological and cytological analysis. There is also the factor of admixture of peripheral blood with aspirated sternal marrow. The ribonucleic acid, however, regularly exceeds the deoxyribonucleic acid, the ratios ranging from 3.8:1 to 1.0:1, with a ratio of 2.0:1 from the means of the normal series for RNAP and DNAP.

Histochemically, the deoxyribonucleic acid is confined to the

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\* Fuller data on these cases have been deposited with the Librarian, General Library, British Museum (Natural History), London, S.W. 1.

nuclear chromatin throughout the development. The ribonucleic acid is richly concentrated in the cytoplasm and nucleoli of the youngest free hæmopoietic cells, the amount diminishing progressively with development to either red cells or granulocytes. The cytoplasmic basophilia of the pro-erythroblast is deeper than that of any marrow cell other than the plasma cell. The granulocytic series is usually less heavily endowed with ribonucleic acid at all stages than the corresponding stages of the red-cell series. In both red-cell and granulocytic series, the basophilia of cytoplasm and nucleoli attributable to ribonucleic acid diminishes with the appearance of hæmoglobin or specific granules. In the earlier stages mitotic division occurs, and the daughter cells show progressive reduction in size in the red-cell series. Cell size increases in the granulocyte series to the myelocyte stage, when mitoses are most frequent, and then diminishes. The disappearance of the nucleolar ribonucleic acid is accompanied by the accumulation of nucleolus-associated chromatin, which stains at least as intensely as the rest of the chromatin with nuclear dyes (Thorell, 1944; White, 1947). The nucleolus-associated chromatin appears to persist after the cell has lost its power to divide, but tends to become obscured by the condensing nuclear chromatin of the erythroblasts, although still visible in the older granulocytes.

It is suggestive that the high ribonucleic-acid content of the cytoplasm and nucleoli of the younger hæmopoietic cells is connected with the ability to pass through a series of mitotic divisions and to elaborate hæmoglobin or the specific granules. Mitosis of hæmocyto blasts is rarely encountered normally, but the incidence of division is greater in the partly differentiated younger members of the two series. The nucleoli of the hæmocyto blasts are large structures, with a high ribonucleic-acid content and slight development of nucleolus-associated chromatin. With development, reduction in ribonucleic-acid content rapidly occurs, and nucleolus-associated chromatin accumulates at the shrinking periphery and persists.

The results for pernicious anæmia show that in 4 out of 5 cases examined a hyperplastic marrow, with many hæmocyto blasts, megaloblasts and giant metamyelocytes, was accompanied by considerably elevated levels of nucleic acids. Ribonucleic acid was particularly raised, being concentrated chiefly in the cytoplasm and nucleoli of the abundant stem cells and young megaloblasts. Treatment with hæmopoietic factors—purified liver extract or folic acid—produced a reticulocyte response with rapid change in the structure of the marrow, excess of normally developing late erythroblasts and normoblasts now predominating, but with hypercellularity similar to that before therapy. In the 3 cases studied during treatment the nucleic acids showed a fall towards more normal levels, and with subsequent improvement in the blood picture the marrow gradually returned to normal cellularity and hæmopoiesis, and the nucleic acids showed an entirely normal distribution.



The considerable fall in nucleic-acid levels during the time of the reticulocyte response appears to be closely related to the great increase in maturity of the marrow. The stem cells fall to within normal limits, presumably dividing and giving rise to normally developing granulocytes and particularly to red cells. The megaloblastic series rapidly disappears and appears to mature through intermediate or normal stages; more important than the mode of disappearance of the megaloblasts is the fact that subsequent generations of red cells arise and mature normally.

One case of pernicious anæmia (case 5) which showed no raised nucleic-acid levels prior to therapy had a slightly hypocellular though megaloblastic marrow. Hyperplasia of the marrow may occur in many severe or long-continued anæmias. Of the refractory anæmias studied, patient E. B. (case 6) showed a hyperplastic megaloblastic marrow with raised RNAP, the anæmia being completely refractory to all therapy. Patient G. E. (case 7) was in slight relapse, the marrow was rich in partly matured erythroblasts and the DNAP showed relatively greater elevation. Patient C. M. (case 8), with macrocytic anæmia, responded to purified liver extract and the cellular immature marrow with intermediate type erythropoiesis returned to normal; this was accompanied by a fall in both nucleic acids to within the normal range. In the case of patient F. H. (case 9), the macrocytic anæmia was accompanied by a very cellular, immature marrow with a mixed type of hæmopoiesis and very high nucleic-acid levels, particularly of RNAP. Folic acid produced a more mature and normal hæmopoiesis and decreased cellularity of the marrow, with a fall of both nucleic acids to normal, but the peripheral blood picture improved only slightly. Reticulocytosis was never more than 12.1 per cent. Purified and crude liver extracts subsequently produced further improvement in the blood picture.

The good correlation between structure and the distribution of nucleic acids is well brought out in the two principal groups of abnormal results. Hyperplasia with immaturity of the marrow is associated with high values for both nucleic acids and more especially for the RNAP. On the other hand, hyperplastic marrows with many cells of medium maturity show a more pronounced elevation of the DNAP. The abnormal values would therefore appear to be related to the distribution of predominant cell types at least as closely as to the increased total number of cells present. This is also borne out by the rapid fall in nucleic-acid levels observed during the reticulocyte response in treated pernicious anæmia: the degree of maturity changes without any obvious change in the total cellularity. The gradual decline in cellularity with recovery is accompanied by relatively slight further change in the nucleic-acid levels towards normal.

## SUMMARY AND CONCLUSIONS

1. The composition of aspirated sternal marrow has been investigated with regard to general histology, cytological distribution and content of ribonucleic and deoxyribonucleic acids by chemical and histochemical methods in (a) a group of 15 normal individuals, and (b) patients suffering from various blood dyscrasias. The investigations have been repeated at intervals during treatment in 3 cases of pernicious anæmia, 2 cases of macrocytic anæmia, 1 case of nocturnal hæmoglobinuria and 1 case of chronic myeloid leukæmia.

2. The accuracy of the methods in relation to the nature of aspirated sternal marrow is discussed.

3. In the abnormal bone marrow the content of both forms of nucleic acid often considerably exceeds the ranges for the normal series.

4. Increase in ribonucleic acid, and in deoxyribonucleic acid to a lesser extent, is particularly related to hyperplasia of the marrow and an increase in number of the more primitive cell types.

5. From cytochemical evidence, the ribonucleic acid is located chiefly in the cytoplasm and nucleoli of the younger free cells and the amount diminishes progressively with maturation.

6. Deoxyribonucleic acid may be elevated to a greater extent than ribonucleic acid in hyperplastic marrows, with predominance of partially mature cells.

7. Increased maturity of the marrow as a result of treatment is associated with a fall in the content of both forms of nucleic acid to within the ranges of the normal series.

8. In pernicious anæmia the fall in nucleic-acid levels is most marked during the reticulocyte response to hæmopoietic substances and is associated with the maturation of primitive cells possessing large nucleoli and basophilic cytoplasm, and with disappearance of the megaloblasts. Return to normal cellularity and architecture is more gradual, and the nucleic acids then come to lie within entirely normal limits.

9. The relationship of the liver principle and of folic acid to the normal maturation of hæmopoietic cells is discussed, with particular reference to the metabolism of ribonucleic acid and to normal mitosis.

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# MELANIN-FORMING EPIDERMAL TUMOURS OF THE SKIN: A STUDY OF 57 PERSONALLY OBSERVED CASES

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(PLATES V-VIII)

IN 1926 Masson revived Soldan's conception of the origin of benign and malignant melanomata of the skin. By means of histological research he showed that these tumours are to be regarded as terminal neuromas of tactile nerves. He identified the pigment-forming cell or melanoblast with the Langerhans cell and assigned to it a nervous origin.

Thus Masson regarded the skin melanomata as neurogenic, a view readily accepted for the choroidal melanomata. Other observations support this view, namely: skin melanomata are frequently associated with neurofibromatous elements in their deeper parts; neither benign nor malignant melanomata ever show the slightest attempt to undergo epidermal differentiation; the diversity of their histological structure is in keeping with a neurogenic theory.

Having accepted Masson's view, it becomes possible to separate from these a group of melanin-forming tumours of epidermal origin which differ widely in morphology and prognosis from the malignant melanomata. This group of tumours has been recognised in France for many years and appears also in German, American and Italian literature. There is singularly little reference to it by British authors and it would seem that British pathologists in general are unfamiliar with the distinction.

In the literature there is extraordinary confusion both of nomenclature and of theories of origin of the pigment-forming cell. Most authors are agreed that the chromatophores of the dermis are wandering phagocytes which have taken up pre-formed melanin. Controversy centres round whether the epidermal cells can form melanin or whether only specialised cells (dendritic in form and usually called melanoblasts) form the pigment, which is then passed on to the epithelial cells of the rete malpighii ("action amboceptrice"—Masson). In our opinion, in the tumours under review, elaboration of pigment by the

TABLE I.—Description of melanin-forming epidermal tumours (all histological types)

Case no. and sex	Site of tumour	Diameter (mm.)	Duration	No recurrence after treatment	Remarks
<b>A. Squamous papilloma</b>					
1 41 F	Neck	6	14 years	...	Many pigmented spots on back and shoulders
2 42 F	...	...	13 "	...	...
3 48 F	...	11	12	...	...
4 54 F	Breast	12	...	...	...
5 55 M	Axilla	10	8-9 years	...	Semi-pedunculated
6 59 M	Pectoral skin	13	...	6 years	Other pigmented spots present from birth
7 60 F	Bridge of nose	1-5	2 years	5 "	Dark and corrugated
8 61 F	Right ear	12	...	...	Several tumours present, one slightly raised
9 62 M	Anterior abdominal wall	7	...	...	...
10 64 M	Face	>11	...	...	Adjacent intra-epidermal basal-cell cancer present as well
11 65 F	Upper eyelid	5	Many years	1 year	Pedunculated
12 66 M	Front of neck	15	...	...	...
13 68 F	Back	14	...	3 years	...
14 68 M	Left side of chest	8	3 months	...	...
15 69 M	...	14	...	...	...
16 72 F	Left side of neck	7	Since birth	1 year	...
17 F	Below clavicle	6	...	...	Areas of transition to basal-cell cancer
18 F	Abdomen	12	...	...	...
19 M	Arm	13	...	...	...
20	...	14	...	...	...
21	...	13	...	...	...
<b>B. Benign calcified epithelioma</b>					
22 28 M	Thigh	...	...	...	...
23 44 M	Abdomen	8	1 year	...	...
<b>C. Basal-cell carcinoma</b>					
24 33 M	Scalp, above right ear	>15	Many years	...	...
25 33 M	Cheek, 1 in. from angle of mouth	11	7 years	4 years	...
26 35 M	Lower abdomen	9	Many years	...	Slight keratinisation

		Whole tumour intra-epidermal											
27	39 F	Right lower part of back	12	...	...	...	...	...	...	...	...	...	...
28	50 F	Skin of breast	11	...	...	...	...	...	...	...	...	...	...
29	53 F	Nose, near inner canthus	6	...	...	...	...	...	...	...	...	...	...
30	53 M	Abdominal wall	10	...	...	...	...	...	...	...	...	...	...
31	53 M	Thigh	7	...	...	...	...	...	...	...	...	...	...
32	53 M	Scalp	11	...	...	...	...	...	...	...	...	...	...
33	54 M	Inner canthus	7	...	...	...	...	...	...	...	...	...	...
34	55 F	Outer side of left leg	12	...	...	...	...	...	...	...	...	...	...
35	55 M	Above right internal con- dyle femur	>14	...	...	...	...	...	...	...	...	...	...
36	56 M	Nose	17	...	...	...	...	...	...	...	...	...	...
37	60 F	Upper eyelid	13	...	...	...	...	...	...	...	...	...	...
38	62 M	Back	>14	...	...	...	...	...	...	...	...	...	...
39	61 F	Left cheek	2	...	...	...	...	...	...	...	...	...	...
40	65 M	Forehead	Biopsy	...	...	...	...	...	...	...	...	...	...
41	65 F	Upper eyelid	5	...	...	...	...	...	...	...	...	...	...
42	66 M	...	8	...	...	...	...	...	...	...	...	...	...
43	68 M	Temple	12	...	...	...	...	...	...	...	...	...	...
44	68 F	Forehead	12	...	...	...	...	...	...	...	...	...	...
45	71 F	Forehead	25	...	...	...	...	...	...	...	...	...	...
46	74 F	Midline of back	4	...	...	...	...	...	...	...	...	...	...
47	80 M	Thigh, close to groin	50	...	...	...	...	...	...	...	...	...	...
48	F	Labium majus	9	...	...	...	...	...	...	...	...	...	...
49	...	Inner canthus	3	...	...	...	...	...	...	...	...	...	...
50	...	Neck	8	...	...	...	...	...	...	...	...	...	...
51	...	...	9	...	...	...	...	...	...	...	...	...	...
D. Squamous carcinoma													
52	43 F	Face	4	...	...	...	...	...	...	...	...	...	...
53	65 F	Leg	>23	...	...	...	...	...	...	...	...	...	...
54*	...	Temporal region	9	...	...	...	...	...	...	...	...	...	...
55*	F	...	11	...	...	...	...	...	...	...	...	...	...
56	...	...	12	...	...	...	...	...	...	...	...	...	...
57	30 F	Front of lower end of thigh	12	...	...	...	...	...	...	...	...	...	...

\* The only two squamous carcinomas not of transition type (neither traced).

neoplastic epidermal cells seems to be in accord with the appearances we have observed.

The present paper is based on the examination of 57 personally observed epidermal melanin-forming tumours occurring in the human subject (table I) and 6 similar tumours induced by skin-painting with 3:4-benzpyrene in the mouse. The human tumours have been collected over a period of some 20 years, partly from routine biopsy and operation material, partly from material sent by other pathologists for an opinion. Thus information as to age, sex, duration, site etc. is sometimes lacking, but table II summarises such information on these points as we have been able to collect. For this purpose the tumours have been divided into two main groups, comprising on the one hand squamous papillomas and on the other basal-cell and squamous cancers.

#### *Clinical aspects*

*Age.* These tumours are most frequently encountered over the age of 50 years. Only 3 of 16 squamous papillomas and 6 of 27 cancers occurred before this age.

*Sex.* The incidence in the sexes is approximately equal.

*Duration.* Nearly all the tumours had been present for one year or more and approximately half had been present for 10 years or longer.

*Site.* Half the tumours (24 out of 46) were situated on the head or neck, the forehead and inner canthus being common sites; approximately a third were on the trunk and a seventh on the limbs, of which all but one were on the lower limbs. One basal-cell cancer was situated on the labium majus.

*Colour.* This was not often recorded, but gradations from black or bluish to pale brown, grey or white were noted. French authors use the term *slaty* (*grisâtre*) as the characteristic colour of the pigment-forming basal-cell cancers.

*After-history.* Information on this point was obtained in 17 cases—6 of squamous papilloma, 9 of basal-cell cancer and 2 of squamous cancer, both of which, however, showed transition to the basal-cell type (table I). No recurrence after treatment was observed during periods ranging from 1 to 13 years in 16 of the 17 cases. In the remaining case (no. 47, table I) there were multiple pigmented spots in the groin, only one of which was excised; during the following 6 years the remaining tumours grew slowly and ulcerated. These could have been removed but the patient refused operation.

#### *Histology*

The tumours may be classified into the types given in table III, but transitions occur, the most difficult to place being those regarded as squamous cancers with transition to the basal-cell type. The melanin can be seen, even when present in small quantity, in sections stained

## MELANIN-FORMING EPIDERMAL TUMOURS



FIG. 1.—Pigment-forming squamous papilloma situated just below the clavicle (case 17). There is surface keratinisation and large masses of keratinous material are present in the deeper parts of the tumour. In the stroma are melanin-containing phagocytes.  $\times 60$ .

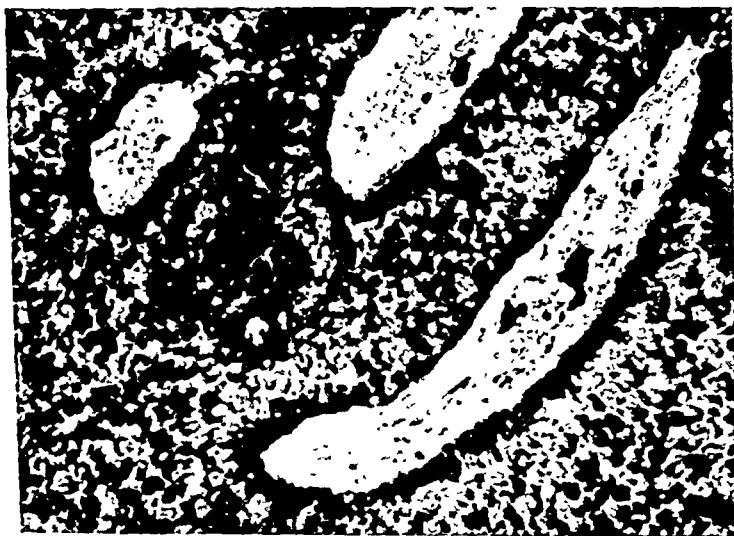


FIG. 2.—Silver staining (Masson's method) of melanin in pigment-forming squamous papilloma of neck (case 12). The most heavily pigmented tumour cells are immediately adjacent to the stroma, in which melanin-containing phagocytes are present.  $\times 230$ .





TABLE II  
Age, sex, duration and site of pigment-forming epidermal tumours

Squamous papillomas										Basal-cell and squamous cancers					
		Stewart and Bonser		Other authors		Total	Per cent.		Stewart and Bonser		Other authors		Total	Per cent.	
Age	20-39 years	0	2	2	2	8.7	5	9	14	18.4					
	40-49 "	3	1	1	4	17.4	1	4	5	6.6					
	50-59 "	3	1	1	4	17.4	9	14	23	30.3					
	60-69 "	9	2	2	11	47.8	9	16	25	33.0					
	Over 70 years	1	1	1	2	8.7	3	6	9	11.8					
	Total	16	7	7	23	...	27	49	76	...					
Sex	Male	8	4	4	12	46.2	14	30	44	55.0					
	Female	11	3	3	14	53.8	15	21	36	45.0					
	Total	19	7	7	26	...	29	51	80	...					
Duration	Present since birth	1	5	5	6	46.2	3	4	7	11.7					
	Present 10 years or more	3	0	0	3	23.1	8	14	22	36.7					
	Present 5-9 years	1	0	0	1	7.7	4	6	10	16.7					
	" 1-4 "	2	1	1	3	23.1	6	10	16	26.7					
	" less than 1 year	0	0	0	0	0.0	4	1	5	8.3					
	Total	7	6	6	13	...	25	35	60	...					
Site	Face and neck	7	2	2	9	40.0	17	46	63	67.7					
	Below clavicle	9*	4	4	13	59.1	13†	17	30	32.3					
	Total	16	6	6	22	...	30	63	93	...					

\* Including 1 on arm.

† Including 5 on lower limb.

by the routine method of hæmatoxylin and eosin. It is, however, much more clearly visible in sections stained by Masson's silver method.

TABLE III

*Histological classification of 57 pigment-forming epidermal tumours*

Group	Type	Number of cases
A	Pigment-forming keratinising squamous papillomas . . . . .	20
	Ditto, with transition to basal-cell cancer . . . . .	1
B	Pigment-forming benign calcified epitheliomas . . . . .	2
C	Pigment-forming basal-cell cancers . . . . .	11
	Ditto, with tendency to keratinisation . . . . .	12
	Intra-epidermal, pigment-forming basal-cell cancers . . . . .	5
D	Pigment-forming squamous cancers . . . . .	2
	Ditto, with transition to basal-cell cancer . . . . .	4
Total . . . . .		57

*Group A. Pigment-forming keratinising squamous papillomas.* The members of this group are often designated acanthomas or hard moles. The tumours are, in fact, well differentiated, benign, keratinising squamous papillomas, though the deeper parts are often very cellular, the cells being oval or spindle-shaped. Keratin is seen as laminæ on the surface and as pearls in the deeper parts (fig. 1). These tumours are usually sessile; only one in this series was pedunculated. In every case the base was flush with the epidermal level. Melanin may be present in the cells of the rete malpighii—especially the basal layer (figs. 2-4), in phagocytes in the stroma, or in the keratin, frequently in all three. The quantity is very variable but in general it is most abundant in the phagocytes. In some tumours there is a transition from heavily pigmented basal cells to more lightly pigmented prickly cells (figs. 2 and 3). In one case (no. 16) transition to basal-cell cancer had occurred, half of the growth lying deep to the general level of the epidermis.

The tumours of this group are well differentiated and histologically benign, but the deeper parts are usually more cellular than in their non-pigmented counterparts. Simple excision is the treatment of choice. Deep to one tumour (no. 1, table I) were many hyperplastic sebaceous glands, the tortuous dilated ducts of which passed through the tumour to the surface and formed some of the keratin-containing cysts seen in the sections. An exactly similar appearance was described by Du Bois (1914-15).

*Group B. Pigment-forming benign calcified epithelioma.* Two examples of this type were encountered. They are not heavily pigmented, but melanin is present in the epithelial cells lining the



## PLATE VI

FIG. 3.—Colour photograph of parallel section to that shown in fig. 2. H. and E.  $\times 230$ .

FIG. 4.—Melanin-containing tumour cells of squamous papilloma of neck (case 16). Note that nearly every cell contains pigment.  $\times 500$ .

FIG. 5.—Benign calcified epithelioma of thigh (case 22), showing melanin-containing epithelial cells.  $\times 500$ .

FIG. 9.—Intra-epithelial basal-cell cancer of back (case 27). Whorled arrangement of pigment-containing tumour cells situated within the epithelium.  $\times c. 180$ .

FIG. 10.—High-power view of same field as fig. 9.  $\times 500$ .

MELANIN-FORMING EPIDERMAL TUMOURS

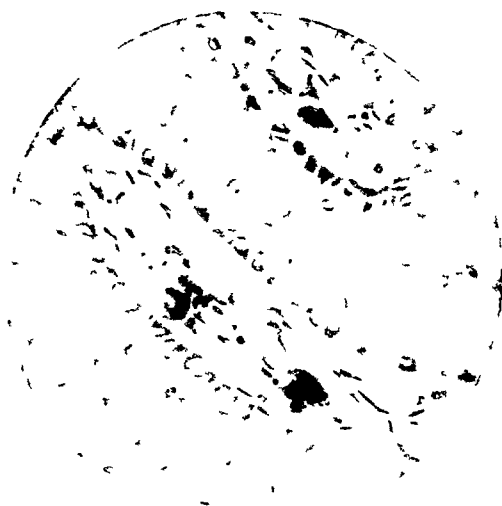


FIG. 3.



FIG. 4

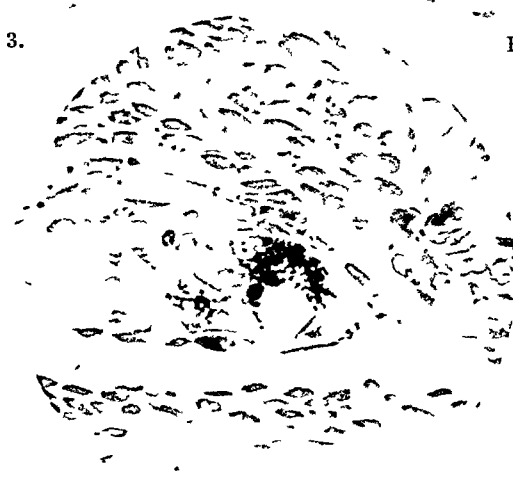


FIG. 5.



FIG. 9.



FIG. 10.

forming variety of rodent ulcer than in its non-pigmented counterpart. In some tumours mitoses are abundant and the branching columns penetrate widely and deeply. Melanin is again present in the epithelial cells, in phagocytes in the stroma (fig. 7) and in the adjacent normal epidermis; in one case melanin was seen in the keratin (fig. 6). As in the group of squamous papillomas, the quantity is very variable. One tumour was composed of the usual branching columns in the dermis and there were in addition areas of intra-epidermal basal-cell cancer in the overlying epidermis (fig. 5).

The pure intra-epidermal type of tumour (5 cases) is rather different in that all the tumour cells lie within the epidermis. One (case 27) measured 12 mm. across and yet none of the tumour cells was outside the confines of the epidermis (figs. 9 and 10). In one case there was an adjacent pigment-forming squamous papilloma. An intra-epidermic melanin-forming basal-cell cancer was described by Jadassohn (1926).

*Group D. Pigment-forming squamous cancers.* All these cases show downgrowth into the dermis except case 55, where the tumour, measuring 11 mm. across, is pedunculated and there is downgrowth only into the pedicle. All are well-differentiated keratinising squamous cancers containing melanin within the epithelial cells, in phagocytes and usually in the keratin (figs. 11 and 12). The most heavily pigmented of all the tumours examined occurred in this group: it was black to the naked eye (no. 54).

*Induced pigment-forming squamous cancers in the mouse.* In the course of observation of 47 squamous epidermal tumours induced in a strain of black mice (IF) with 3:4-benzpyrene, it was noticed that 6 were melanin-forming. These tumours do not differ histologically from other squamous cancers induced by this means in mice of any coat colour, except that the basal epithelial cells, phagocytes and keratin all contain melanin (fig. 13). This means of induction of pigment-forming epidermal tumours should be valuable in the study of the pigment-forming mechanism by the dopa reaction and other methods.

### Discussion

In 1935, Touraine described for the first time a group of plaster casts made at the St Louis Hospital, Paris, between the years 1875 and 1919 depicting 12 pigmented epithelial tumours situated in various parts of the body. He also collected 42 cases from the literature. Uncoloured photographs of 3 of the casts show very beautifully three types of melanin-forming basal-cell cancer—flat, exuberant and ulcerated with beaded margin. It is evident that the staff of the hospital had been familiar with the distinction between the melanin-forming epidermal tumours and the melanomata for many years. Listed in table IV are all the cases of melanin-forming squamous papilloma and benign calcified epithelioma which we have encountered

## MELANIN-FORMING EPIDERMAL TUMOURS

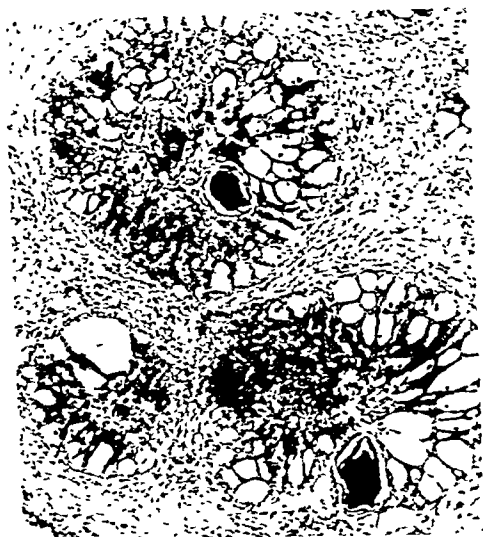


FIG. 6.—Pigment-forming basal-cell cancer of abdominal wall of cylindromatous or cribriform type (case 30). Three well-defined tumour areas are present in a loose fibrous stroma. Abundant melanin is present, in the tumour cells, the intrinsic stroma of the tumour nodules and two eccentric keratinous whorls.  $\times$  c. 30.

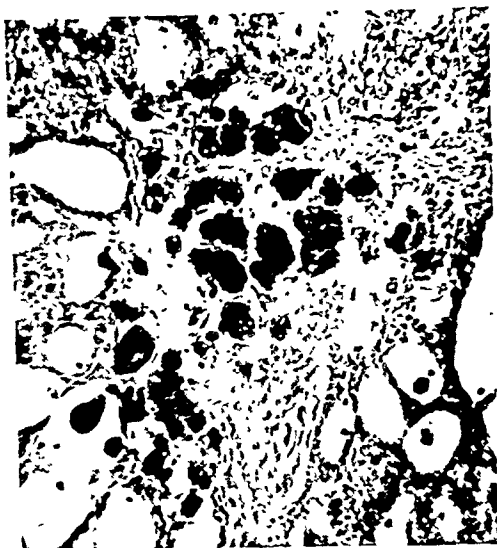


FIG. 7.—High-power view of melanin-containing phagocytes in the intrinsic stroma of the same tumour as fig. 6.  $\times$  c. 120.



FIG. 8.—Pigment-forming basal-cell cancer of forehead (case 40). Above is the epidermis covering the tumour. In it there is intra-epidermal spread of pigmented tumour cells. Immediately beneath the epithelium are irregular pale islands of stroma in which are a few melanin-containing phagocytes and some small groups of tumour cells. Beneath this stroma is the main mass of pigment-containing tumour cells of spindle shape; below there are numerous melanin-containing phagocytes in an area of degenerate tumour tissue.  $\times$  100.





MELANIN-FORMING EPIDERMAL TUMOURS

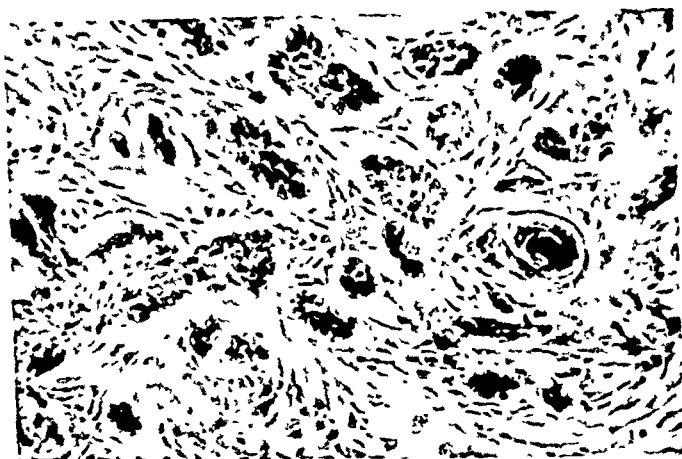


FIG. 11. — Pigment-forming squamous cancer of leg (case 53). One keratinising cell-nest shows melanin in the keratin.  $\times 230$ .

FIG. 12. — Same tumour as fig. 11. Melanin in the tumour cells and in a large keratinous focus (top right).  $\times 140$ .

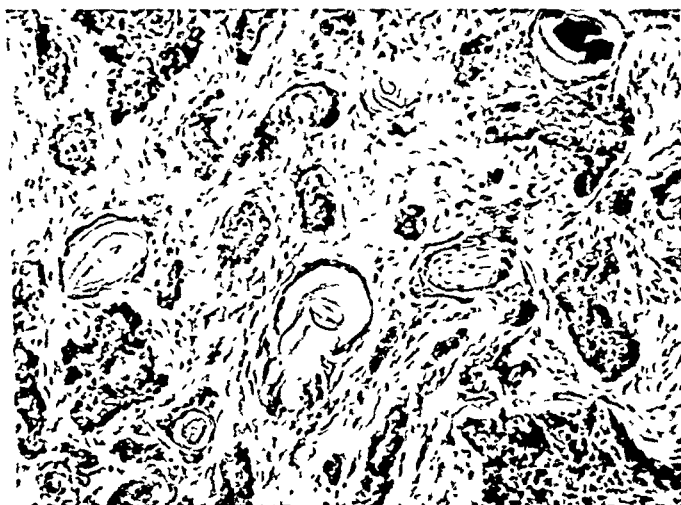


FIG. 13. — Induced pigment-forming squamous cancer in male mouse of IF strain painted with 0.3 per cent. 3:4-benzpyrene in benzene for 36 weeks; in the centre, a whorled mass of keratin and around it melanin-containing tumour cells.  $\times 140$ .



in a fairly considerable search of the literature. We have also collected 57 cases of melanin-forming basal-cell carcinomas described by other authors (see "Additional references"). A list of these has been lodged with the Librarian, General Library, British Museum (Natural History), London, S.W. 7.

Perhaps the earliest reference is that of Pollio (1906), who described 3 histological types of pigmented tumour in a case of von Recklinghausen's disease, the last group comprising pigmented spots without cell nests but with acanthosis. No illustrations are given and it is not certain that the acanthotic hard mole is meant. Seven pigmented basal-cell cancers were mentioned by Owen (1930), without further description, as occurring in a series of 836 surgically removed cancers of this type at the Mayo Clinic. Dawson (1925) studied 37 examples of acanthotic mole and regarded them as similar in origin to the melanomas (which in his view are derived from the epidermis) except that (p. 540) "cell proliferation and cell pigmentation take place without cell detachment", thus explaining, in Dawson's view, the absence of nævus cells in the corium. Only one of Dawson's tumours of this group showed transition to malignancy. He stated (p. 537) that these tumours often occur in clusters in the same region, *e.g.* in the zones of the intercostal nerves. Bloch (1927) described 4 melanin-forming epidermal tumours which he classified as benign or at most pre-cancerous; these, from the illustrations, are of the type we have called pigment-forming basal-cell cancers.

The age, sex, duration and site of the tumours described by other authors are in close accord with what we have observed in our own series (table II). Touraine (p. 790) states that "*le siège des épithéliomas pigmentés est indifférent,*" but when our own cases are added to those of all other authors there is no doubt that these tumours occur with greatest frequency on the head and neck. This fact may explain the experience of many radiologists that pigmented tumours of the head and neck respond more readily to radiation therapy than those of other sites.

There is considerable difference of opinion as to the prognosis. Schröpl (1927), Touraine (1935) and Gaté *et al.* (1937) regard the prognosis as the same as that of the non-pigmented counterparts, a view in which we concur. Halkin (1937), however, thinks that the pigmented forms are probably more dangerous. The reason for this view is his earlier observation that some of these tumours may develop at the site of previously present pigmented nævi (meaning benign melanomata). We are inclined to think that he may have been confusing chromatophores with nævus cells. Touraine uses the paler colour and slower growth as the best clinical means of differentiation from the true melanomata.

The complexity of the views of other authors on the question of the origin of the pigment has already been mentioned. Masson thinks that only specialised cells of neurogenic origin can form melanin,

whereas Bloch thinks that all basal epithelial cells can form pigment when stimulated functionally. Thus some authors, notably Touraine and Halkin, regard the presence of pigment within the tumour cells as due to the proximity of the latter to the epidermis, which feeds the tumour cells with pigment. Therefore, where ulceration has removed the surface epidermis, these authors would expect to find the tumour cells lacking in pigment and this they claim has generally been the case. Similarly, they have found the deeper parts of the tumours to be less pigmented than the more superficial parts. Gaté *et al.* state that pigmentation does not occur in ulcerated tumours. There is no evidence in our material that the presence of pigment in the tumour cells is dependent upon the presence of an intact surface epithelium, nor have we observed that the deeper parts of the tumours are less pigmented than the more superficial. All the appearances go to show that the tumour cells form the pigment.

In addition to the cases in the literature in which a single tumour or only small numbers of pigment-forming epidermal tumours are described, there are cases reported with multiple lesions, sometimes exceeding 200 in number and situated in all sites, but especially on the face, neck and back, of which one or more tumours proved on histological examination to be pigmented squamous papillomas or pigmented basal-cell cancers (table V). We have not encountered any such case in our series.

Pigmented basal-cell cancers have also been described in relation to xeroderma pigmentosum (Kreibich, 1901; Pollitzer, 1905). Blumenthal (1931-32) described a case of long-standing psoriasis, treated on more than 200 occasions with X-rays and given large doses of arsenic over a long period so that there was ultimately generalised darkening of the skin, from which a pigment-forming basal-cell cancer was excised. It is impossible to determine whether this tumour merely participated in the general arsenical changes.

### *Summary*

A series of 57 epidermal pigment-forming tumours of the skin in man is described. They are classified as squamous papillomas (21), benign calcified epitheliomas (2), basal-cell carcinomas (28) and squamous carcinomas (6).

These tumours have nothing in common with the malignant melanoma of the skin or its benign counterpart, except that they are pigment-forming. Clinically they behave like the corresponding non-melanin-forming epidermal tumours, being simple, locally malignant or frankly malignant as the case may be.

The literature has been scrutinised for descriptions of similar tumours and the results analysed.

Six examples of melanin formation in epidermal tumours induced by painting the skin of the mouse with 3 : 4-benzpyrene are described.

TABLE V  
Cases of multiple pigment-forming epidermal tumours described by other authors

Author	Year of publication	Age and sex	Site of tumours	Duration	Remarks
Dawson	1925	75 F	Large number all over trunk	Many years	5 biopsies made; all tumours were pigmented "neurothotic naevi"
Pautrier and Archambault	1927	53 F	Face, neck, nape of neck, sternum, abdomen, pubis, back and thighs	22 years (now tumours appearing all the time)	5 biopsies made; one was pigmented basal-cell cancer, apparently intra-epidermal
Montgomery	1929	50 F	Left breast, chest, abdomen, arms, chin, face and neck	15 years	Biopsy of tumour of skin of left breast showed pigmented squamous or basal-cell cancer. Author also collected 17 other cases ranging from 28 to 60 years, in all, 10 in females and 8 in males, some with over 200 tumours on the trunk. Not all the tumours were pigment-forming
Nomland	1929	71 M	Face, sternum, back and other areas	5 years	Biopsy of tumour of groin showed pigmented basal-cell cancer
"	1932	38 F	Face and neck	As long as she could remember	4 biopsies made; 2 were basal-cell and 2 squamous cancers, all with pigment
Milian <i>et al.</i>	1932	37 F	Cheeks, neck and breast	Since birth	5 biopsies made; one was a pigmented squamous papilloma of breast skin, one a pigmented basal-cell cancer of cheek
Weill	1934	34 F	Forehead, upper lip, eyelids, cheeks, nape of neck and trunk	14 years	Basal-cell cancer. Pigment not mentioned but Pautrier thought this was similar to his case
Vigno	1936	22 F	Face, nape of neck, sacro-iliac region, thorax, abdomen, legs and hands	10 years (now tumours appearing all the time)	6 biopsies made; 2 were pigmented basal-cell cancers; the others were non-pigmented but basal-cell
Nisbet	1943	24 F	Face and back	Since birth (now tumours appearing for 12 years)	2 biopsies made; both pigmented basal-cell cancers

We are especially indebted to Dr J. T. Ingram for affording us access to his clinical notes. To many other colleagues and in particular to Professor Cyril Polson we are also very grateful.

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576 . 851 . 49 (*Salmonella chittagong*)

## A NEW SALMONELLA TYPE: *SALMONELLA* *CHITTAGONG*

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THE *Salmonella* here described was isolated from the stools of a West African cook in Chittagong, Assam, South-Eastern Asia Command, during the course of a routine examination for enteric carriers and was sent by Major J. A. Boycott to each of our laboratories as an unusual salmonella for identification. Isolation was on S.S. agar (Difco), the colony picked for investigation being small, circular and opaque, with a black centre. It was tested for purity on MacConkey's agar before initial biochemical and serological examination.

### CULTURAL AND BIOCHEMICAL FINDINGS

The organism (no. 21340) was a motile Gram-negative bacillus, producing colonies on nutrient agar indistinguishable from those of salmonellæ. Acid and gas were produced in glucose, maltose, mannitol, dulcitol, arabinose, rhamnose, lævulose, inositol, sorbitol, xylose and mannose. Lactose, sucrose, salicin, inulin and raffinose were not fermented. Indole and acetylmethylcarbinol were not produced; the methyl-red test was negative, the organism grew in Koser's citrate medium, and gelatin was not liquefied.

### SEROLOGICAL ANALYSIS

#### *Somatic antigens*

Preliminary slide agglutination showed that the new organism, subsequently referred to as *Salm. chittagong*, was agglutinated by O sera III.X.XXVI, I.III.XIX and III.XV; in tubes it was agglutinated by an absorbed serum containing only factors X.XXVI

and by another containing only factor XIX but not by one containing only factor XV. It was therefore thought that it probably contained factor III, together with other antigens present in O sera prepared with *Salm. london* (III.X.XXVI) and *Salm. senftenberg* (I.III.XIX). Further work supported this view, as in order completely to remove the homologous agglutinins from a serum prepared with *Salm. chittagong*, an absorbing suspension containing both *Salm. london* and *Salm. senftenberg* was required; neither alone was effective. In order to prove this hypothesis the individual factors of the somatic complex were investigated.

TABLE I

Suspension	<i>Salm. chittagong</i> serum dilutions									
	10	20	50	100	200	400	800	1600	3200	Saline control
<i>Salm. chittagong</i> (O)	++++	++++	++++	++++	++++	++++	+++	++	—	—
<i>Salm. london</i> (O)	++++	++++	++++	++++	++++	++++	++++	++	—	—
<i>Salm. newington</i> (O)	++++	++++	++++	++++	++++	++++	±	—	—	—
<i>Salm. minnesota</i> (O)	++++	++++	++++	++++	++++	++++	±	—	—	—
<i>Salm. senftenberg</i> (O)	++++	++++	++++	++++	++++	++++	++	—	—	—
<i>Salm. paratyphi</i> A (O)	+	±	—	—	—	—	—	—	—	—

++++ complete agglutination

+++ agglutination not quite complete

++ well-marked agglutination without sedimentation

+ trace visible to naked eye

± trace visible only with lens

TABLE II

Suspension	<i>Salm. senftenberg</i> serum dilutions							
	50	100	200	400	800	1600	3200	Saline control
<i>Salm. chittagong</i> (O)	++++	++++	++++	+++	—	—	—	—
<i>Salm. london</i> (O)	++++	++++	++++	+++	—	—	—	—
<i>Salm. anatum</i> (O)	++++	++++	++++	+++	±	—	—	—
<i>Salm. senftenberg</i> (O)	++++	++++	++++	++++	++++	+++	—	—
<i>Salm. newington</i> (O)	++++	++++	++++	+++	+	—	—	—
<i>Salm. paratyphi</i> A (O)	+++	++	—	—	—	—	—	—

TABLE III

Suspension	<i>Salm. london</i> serum dilutions							
	50	100	200	400	800	1600	3200	Saline control
<i>Salm. chittagong</i> (O)	++++	++++	++++	++++	+++	+	—	—
<i>Salm. london</i> (O)	++++	++++	++++	++++	+++	±	—	—
<i>Salm. senftenberg</i> (O)	++++	++++	++++	++++	+++	—	—	—
<i>Salm. newington</i> (O)	++++	++++	++++	++++	+++	—	—	—

Tables I-III show the serological relationship between *Salm. chittagong* (O) and organisms of the III.X.XXVI group (*Salm. london*), the I.III.XIX group (*Salm. senftenberg*), and the III.XV group (*Salm. newington*). Only factor III is common to these three groups of organisms.

Table IV gives the result of the absorption of *Salm. senftenberg* (O) antiserum with *Salm. chittagong*, proving that the factor common to *Salm. chittagong*, *Salm. london* (III.X.XXVI) and *Salm. newington* (III.XV), has been removed completely and the titre to the homologous strain reduced by 75 per cent. These results establish the presence of factor III in *Salm. chittagong*.

TABLE IV

Suspension	<i>Salm. senftenberg</i> (O) serum absorbed with <i>Salm. chittagong</i>						
	25	50	100	200	400	800	Saline control
<i>Salm. chittagong</i> (O) . .	—	—	—	—	—	—	—
<i>Salm. london</i> (O) . .	—	—	—	—	—	—	—
<i>Salm. senftenberg</i> (O) . .	++++	++++	++++	++	+	—	—
<i>Salm. newington</i> (O) . .	—	—	—	—	—	—	—
<i>Salm. paratyphi</i> A (O) . .	—	—	—	—	—	—	—

Reference to tables I-III suggests that, while there can be a single factor common to all the groups, there is the possibility of a relationship between other factors of the new strain and of any single group.

Proof that the formula was some combination of the III.X.XXVI and I.III.XIX complexes was afforded by absorption of *Salm. chittagong* antiserum with *Salm. senftenberg* (table V), followed by absorption with *Salm. anatum* (table VI). Similar results were obtained when the order of the absorbing suspensions was reversed.

TABLE V

Suspension	<i>Salm. chittagong</i> serum absorbed with <i>Salm. senftenberg</i>			
	25	50	100	Saline control
<i>Salm. chittagong</i> (O) . .	++++	++	—	—
<i>Salm. anatum</i> (O) . .	++	—	—	—
<i>Salm. senftenberg</i> (O) . .	—	—	—	—
<i>Salm. london</i> (O) . .	++	—	—	—
<i>Salm. newington</i> (O) . .	—	—	—	—

TABLE VI

Suspension	<i>Salm. chittagong</i> serum absorbed with <i>Salm. senftenberg</i> + <i>Salm. anatum</i>		
	25	50	Saline control
<i>Salm. chittagong</i> (O) . .	—	—	—
<i>Salm. anatum</i> (O) . .	—	—	—
<i>Salm. senftenberg</i> (O) . .	—	—	—
<i>Salm. london</i> (O) . .	—	—	—

A *Salm. anatum* antiserum containing agglutinins to factors III and X only was absorbed with *Salm. chittagong*, which removed all

the homologous agglutinins, thus proving the presence of factor X (table VII).

TABLE VII

Suspension	<i>Salm. anatum</i> serum absorbed with <i>Salm. chittagong</i>		
	25	50	Saline control
<i>Salm. chittagong</i> (O) . .	—	—	—
<i>Salm. anatum</i> (O) . .	—	—	—
<i>Salm. senftenberg</i> (O) . .	—	—	—
<i>Salm. london</i> (O) . .	—	—	—

Absorption of *Salm. chittagong* serum with *Salm. senftenberg* (table V) greatly diminished the titres to the homologous strains and to organisms of the III.X.XXVI group. The residual agglutinins might represent factor X or factor XXVI, or both. However, it has been proved that factor X is present in *Salm. chittagong*. It remained therefore to establish whether or not factor XXVI was also present. An antiserum prepared against *Salm. minnesota* (O) (XXI.XXVI) agglutinated *Salm. chittagong* (O), though to a lower titre than other salmonellæ containing factor XXVI (table VIII).

TABLE VIII

Suspension	<i>Salm. minnesota</i> (O) serum dilutions								Saline control
	10	20	40	80	160	320	640	1280	
<i>Salm. chittagong</i> (O)	++++	+++	++	+	—	—	—	—	—
<i>Salm. london</i> (O)	++++	++++	++++	++++	++++	++++	++++	—	—
<i>Salm. minnesota</i> (O)	++++	++++	++++	++++	++++	++++	++++	—	—
<i>Salm. anatum</i> (O)	++++	++++	++++	++++	++++	++++	++	—	—

Absorption of *Salm. minnesota* serum with *Salm. london* removed all agglutinins to *Salm. london*, *Salm. anatum* and *Salm. chittagong* (table IX).

TABLE IX

Suspension	<i>Salm. minnesota</i> serum absorbed with <i>Salm. london</i>							
	10	20	40	80	160	320	640	Saline control
<i>Salm. chittagong</i> . .	—	—	—	—	—	—	—	—
<i>Salm. london</i> . .	—	—	—	—	—	—	—	—
<i>Salm. minnesota</i> . .	++++	++++	++++	++++	++	—	—	—
<i>Salm. anatum</i> . .	—	—	—	—	—	—	—	—

Since the initial titre against *Salm. chittagong* was only 1:40, the proof of the presence of factor XXVI in this strain was not convincing. Absorption of *Salm. minnesota* antiserum with *Salm. chittagong* reduced the titre of the serum to all strains containing factor XXVI, with a 90 per cent. loss for *Salm. london* and *Salm. anatum* and a

75 per cent. loss for the homologous strain (table X), thus proving the presence of factor XXVI in *Salm. chittagong*.

TABLE X

Suspension	<i>Salm. minnesota</i> (O) serum absorbed with <i>Salm. chittagong</i>							
	10	30	60	120	240	480	960	Saline control
<i>Salm. chittagong</i> (O)	—	—	—	—	—	—	—	—
<i>Salm. london</i> (O)	+++	+++	+++	+++	+++	+++	—	—
<i>Salm. minnesota</i> (O)	+++	+++	+++	+++	+++	+++	—	—
<i>Salm. anatum</i> (O)	+++	+++	+++	—	—	—	—	—

Factors III.X and XXVI are present in *Salm. chittagong*, but absorption of *Salm. chittagong* serum was complete only with a mixture of III.X.XXVI and I.III.XIX. It remained to ascertain which components of the I.III.XIX group were present in addition to factor III. Absorption of *Salm. chittagong* antiserum with *Salm. anatum* or *Salm. london* left an appreciable titre to *Salm. senftenberg* (tables XI and XII).

TABLE XI

Suspension	<i>Salm. chittagong</i> serum absorbed with <i>Salm. anatum</i>				
	25	50	100	200	Saline control
<i>Salm. chittagong</i> (O)	+++	+++	+	—	—
<i>Salm. anatum</i> (O)	—	—	—	—	—
<i>Salm. senftenberg</i> (O)	+++	+++	+++	—	—
<i>Salm. london</i> (O)	—	—	—	—	—

TABLE XII

Su-pension	<i>Salm. chittagong</i> serum ab-orbed with <i>Salm. london</i>							
	10	20	40	80	160	320	640	Saline control
<i>Salm. chittagong</i> (O)	+++	+++	+++	+++	+	—	—	—
<i>Salm. london</i> (O)	—	—	—	—	—	—	—	—
<i>Salm. senftenberg</i> (O)	+++	+++	+++	+++	+++	—	—	—
<i>Salm. paratyphi</i> A (O)	—	—	—	—	—	—	—	—

This residual titre could be due to agglutinins to factor I or factor XIX, or both. It will be seen (table XIII) that *Salm. paratyphi* A antiserum agglutinates *Salm. chittagong*, and (table I) that *Salm. chittagong* serum agglutinates *Salm. paratyphi* A.

TABLE XIII

Suspension	<i>Salm. paratyphi</i> A (O) serum dilutions									
	10	20	40	80	160	320	640	1280	2560	Saline control
<i>Salm. chittagong</i> (O)	++	+	—	—	—	—	—	—	—	—
<i>Salm. paratyphi</i> A (O)	++++	++++	++++	++++	++++	+++	++	+	—	—
<i>Salm. senftenberg</i> (O)	++++	++++	++++	++++	+++	+++	+++	+++	+	—

Absorption of *Salm. paratyphi* A antiserum with *Salm. chittagong* markedly reduced the titre of this serum to both *Salm. paratyphi* A and *Salm. senftenberg* (table XIV).

TABLE XIV

Suspension	<i>Salm. paratyphi</i> A serum absorbed with <i>Salm. chittagong</i>							
	10	20	40	80	160	320	640	Saline control
<i>Salm. chittagong</i> (O) .	—	—	—	—	—	—	—	—
<i>Salm. senftenberg</i> (O) .	++++	++++	++++	++	+	—	—	—
<i>Salm. paratyphi</i> A (O) .	++++	++++	++++	++++	++	+	—	—

Agglutination of *Salm. senftenberg* by *Salm. paratyphi* A antiserum is due to factor I, which is common to both organisms. This reduction in titre to *Salm. senftenberg* was explained by the presence of factor I in *Salm. chittagong*, but since the absorption was not complete it was concluded that factor I exists in the partial state in *Salm. chittagong*.

Preliminary absorption of *Salm. chittagong* antiserum with *Salm. london* (table XII) followed by *Salm. paratyphi* A (table XV) shows that the residual titre to *Salm. senftenberg* is unaltered by the second absorption.

TABLE XV

Suspension	<i>Salm. chittagong</i> serum absorbed with <i>Salm. london</i> + <i>Salm. paratyphi</i> A						
	10	20	40	80	160	320	Saline control
<i>Salm. chittagong</i> (O) . .	++++	++++	++++	+++	+	—	—
<i>Salm. london</i> (O) . .	—	—	—	—	—	—	—
<i>Salm. senftenberg</i> (O) . .	++++	++++	++++	++++	++	—	—
<i>Salm. paratyphi</i> A (O) . .	—	—	—	—	—	—	—

These absorptions removed all agglutinins to factors I, III, X and XXVI. The residual titre to *Salm. senftenberg* must therefore be due to factor XIX, since all homologous agglutinins are removed from *Salm. chittagong* antiserum by absorption with *Salm. london* followed by *Salm. senftenberg*. Absorption of *Salm. senftenberg* antiserum with *Salm. chittagong* removed all factor III and factor I (table IV). A residual titre remained to *Salm. senftenberg*. This could only represent factor XIX. It was therefore concluded that this factor is present in the incomplete state.

The somatic complex of this organism is therefore (I).III.X.(XIX).XXVI.

#### Flagellar antigens

Formolised broth cultures of *Salm. chittagong* were agglutinated by *Salm. paratyphi* B (H, phase 1) antiserum to its titre but by no other flagellar antiserum. Similarly, antiserum produced to *Salm. chittagong*

The somatic component of this second flagellar phase was shown to be identical with that of the alternate phase. Of a large number of H antisera tested, *Salm. chittagong* (phase 2) was agglutinated to a significant titre only by *Salm. paratyphi* A (H) serum. The serological reactions of *Salm. chittagong* (H, phase 2) and its homologous anti-serum are shown in table XVI.

H antiserum	Homologous titre	H suspension	Titre
<i>Salm. paratyphi</i> A (C.M.P.L.*)	1:250	21340 (H, phase 2)	1:10
<i>Salm. paratyphi</i> A (Oxford standard)	1:250	21340 (H, phase 2)	1:125
<i>Salm. paratyphi</i> A (Dr Scott)	1:16,000	21340 (H, phase 2)	1:200
<i>Salm. paratyphi</i> A (C.M.P.L.)	1:250	21340 (H, phase 2)	NH 1:10
" " " " " " " " " " " " " "	1:500	21340 (H, phase 2)	NH 1:25
" " " " " " " " " " " " " "	1:20,000	21340 (H, phase 1)	1:2000
No. 21340 (phase 2) (Salm. Ref. Lab.)	1:25,600	<i>Salm. paratyphi</i> A	1:10,000
		<i>Salm. paratyphi</i> B (phase 1)	1:600
		<i>Salm. paratyphi</i> A	1:1600

Results of absorption tests, employing a suspension of naturally acquired phase 2 organisms only, are given in table XVII.

H antiserum	Absorbing suspension	Titre against	
		<i>Salm. paratyphi</i> A (H)	No. 21340 (H, phase 2)
<i>Salm. paratyphi</i> A (Oxford standard)	Unabsorbed	25,600	6400
No. 21340 (phase 2)	No. 21340 (phase 2)	25,600	NH 1 : 100
	Unabsorbed	1600	25,600
	<i>Salm. paratyphi</i> A (H)	NH 1 : 100	12,800

It is clear from these experiments that *Salm. chittagong* (H, phase 2) has only minor antigens in common with *Salm. paratyphi* A (H).



*Salm. chittagong* (H, phase 2) antiserum, absorbed with a suspension of *Salm. paratyphi* A (H)+B (H) and having a homologous titre of 1:12,800, was then tested in 1:100 dilution against a series of *Salmonella* (H) suspensions covering among them all the flagellar antigens hitherto described. *Salm. poona* (H) (z) was agglutinated, the titre of the serum for this type being 1:800. On absorption of phase 2 serum with *Salm. poona* (H) (z) the homologous titre was reduced from 1:12,800 (standard) to 1:12,800 (trace), showing that the reaction was due to minor antigenic similarities. This was further proved by the fact that *Salm. poona* (H) (z) serum, homologous titre 1:32,000, agglutinated *Salm. chittagong* to 1:200 only. It was concluded that phase 2 was not present in any salmonella previously described. Therefore it is designated  $z_{35}$ .

### DISCUSSION

*Salm. chittagong* is of interest on account both of its somatic antigenic structure and of its second flagellar phase. The somatic structure contains all the antigens of *Salm. anatum* and *Salm. senftenberg*. P. R. Edwards, in a personal communication, informs us that he has found three strains of the paracolon group of organisms, isolated from snakes, having flagellar antigens closely related to phase 2 of *Salm. chittagong*. It is possible that strain *Salm. chittagong* may have originated from a snake, because West Africans ate these reptiles, which were not uncommonly found in their kitchens in Assam.

The antigenic formula of *Salm. chittagong* is, therefore: (I).III.X.(XIX).XXVI;  $b \longleftrightarrow z_{35}$ .

No evidence as to the pathogenicity of *Salm. chittagong* for man is available. 0.5 ml. of an 18-hour broth culture of *Salm. chittagong* was inoculated intraperitoneally into each of three mice. These died after 1, 8 and 20 days respectively and *Salm. chittagong* was recovered from cultures of the heart blood and spleen of each mouse.

### SUMMARY

1. A new salmonella type has been isolated from the stools of a West African army cook in the course of a routine examination for enteric carriers.

2. The antigenic formula of this type is (I).III.X.(XIX).XXVI;  $b \longleftrightarrow z_{35}$ , " $z_{35}$ " being an antigen not hitherto described.

3. It is proposed that the new type should be known as *Salm. chittagong*.

We wish to record our indebtedness to Dr F. Kauffmann, State Serum Institute, Copenhagen, and Dr P. R. Edwards of the Department of Animal Pathology, University of Kentucky, for their interest in the work on *Salm. chittagong*; and to Major J. A. Boycott, R.A.M.C., by whom the strain was originally isolated.

# AN ANALYSIS OF CERTAIN FACTORS ASSOCIATED WITH THE PRODUCTION OF EXPERIMENTAL DISSECTION OF THE AORTIC MEDIA, IN RELATION TO THE PATHOGENESIS OF DISSECTING ANEURYSM

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IN a small series of observations made for inclusion in a recent clinical and pathological review of some cases of dissecting aneurysm of the aorta (Halliday and Robertson, 1946) one of us (J. S. R.) noted that remarkably high pressures (of the order of 600 mm. Hg. and often more) were required to produce artificial dissection of the aortic media in the cadaver. The number of aortas examined at that time was too small to allow any conclusions to be drawn. It seemed worth while, however, considerably to extend our observations, in the hope that statistical analysis of a much larger body of data might allow sex or age differences to emerge, or evidence as to the relative resistance to dissection of different regions of the aorta and on the influence of other factors enumerated below. The present paper reports the results of such an analysis of 42 cases.

Since the pioneer work of Nicholls (1727-28, Burns (1809) and Peacock (1843), several papers have appeared in which the possible mechanical factors operating in cases of dissecting aneurysm of the aorta have been the subject of experimental enquiry. These are reviewed in the monographs of Shennan (1934) and Sailer (1942). Much of this work was done many years ago, and only occasional or at the most small numbers of vessels were examined. The interest usually centred on the pressure required to burst the aorta as a whole (Oppenheim, 1918; Klotz and Simpson, 1932; Moritz, 1932; Schnurbein, cited by Sailer, 1942) or on the nature and course of dissections produced mechanically by forcing fluid into the vessel after mural damage of various types had been produced (Peacock). We have found no references to studies based on larger numbers of aortas, nor to the degree of pressure needed to produce the actual dissection and splitting apart of the planes of the tunica media. It is this aspect of the problem which we have studied in the present investigation; that is, we have measured the head of pressure required to cause fluid injected into the media to burrow its way through its substance. At

the same time notice has been taken of other possibly associated factors, namely age, sex, the length of time between the death of the subject and the taking of the measurements, the degree of associated atheroma and the presence or absence of arterial hypertension and hypertrophy of the left ventricle, as well as other factors considered relevant in individual cases.

#### *Materials and methods*

Adult subjects coming to autopsy in the Royal Prince Alfred Hospital, Sydney, provided the material. All bodies were stored in a refrigerating chamber at 40° F. before autopsy. The period of refrigeration was somewhat less than the number of hours since death, the difference being the time between death and the carriage of the body to the hospital mortuary. The cases were not consecutive, but aortas were studied as often as the work of a large routine and teaching department permitted.

After removal from the body the aorta was washed briefly. Nine pieces, each approximately 2 cm. square, were cut from it, three from each of three regions, namely the ascending (intrapericardial) portion, the arch, and the descending (abdominal) portion. Areas of obvious atheroma were avoided. A hypodermic needle connected by pressure tubing to a syringe filled with water and also to a mercury manometer so arranged that the height of the mercury meniscus measured the pressure at the point of the needle was then carefully inserted, parallel to the intimal surface, into the media of the piece of aorta under investigation, approximately to the centre of the piece. Great care was taken not to allow the needle, in its passage from the edge to the centre, to pass out of the medial coat into the intima or adventitia. The pressure in the system was then slowly increased and the pressure noted at which a bleb of water formed at the needle point, pushed out pseudopodia-like projections and spread through the substance of the media. This end-point was usually sharp, and readings were possible to an estimated accuracy of  $\pm 15$  mm. Hg. The measurements were thus "acute" readings and did not take into account the fatigue, in the physical sense, of the structures under stress. The degree of atheromatous disease affecting the various regions of the aorta was then noted according to a system of scoring in which the symbols O (none), M (moderate), + and ++ represented increasing degrees of severity. In a similar way the degree of hypertrophy of the left ventricle of the heart was recorded as (-) (smaller than usual); N (normal in size); + (moderate hypertrophy) and ++ (considerable hypertrophy). The sex, age, number of hours since death, blood pressure records and heart weight were also noted.

#### *Analysis of results*

The detailed findings in 42 cases are given in a table which has been deposited with the Librarian, General Library, British Museum (Natural History), London, S.W. 7. Some statistical averages derived from this table of original data are set out in table I.

It was found that there was very considerable variation between individual measurements, even those made on pieces cut from the same region of the one vessel. In spite of this all measurements represented high pressures. The lowest figure recorded was 230 mm. for the pressure at which dissection of the media occurred (hereafter termed the dissecting pressure). This occurred in one portion of the

descending aorta in case 16, a male aged 68 years who had died from rupture of a saccular aneurysm of the abdominal aorta. The dissecting pressures of the two other pieces of abdominal aorta in this man were 465 and 480 mm. Hg. The highest dissecting pressure of the whole series was 975 mm. Hg. in the arch of case 6, a diabetic male aged 44 years, while the grand mean was 566 mm. Hg. The inference to be drawn from this is that the normal aortic media is well able to withstand the aortic blood pressure should the blood contained in

TABLE I

*Means of individual observations*

	Age (years)	Time since death (hours)	Heart weight (g.)	Dissecting pressure (mm. Hg)			
				Ascending aorta	Arch of aorta	Descending aorta	Combined
Mean	54.2	17.5	372.0	625.0	564.0	509.0	566.0
Standard deviation	14.2	8.4	137.2	103.0	85.0	74.2	69.6
Standard error of mean	2.2	1.3	21.2	16.1	13.3	11.6	10.7

the vessel gain access to the media through any local intimal defect. This inference is strengthened by the fact that these measurements were made on flat unstretched pieces of aorta, while in life the aortic wall is subjected to a distending pressure equivalent to 120-150 mm. Hg., which would tend to resist dissection. These results confirm, from a different aspect, the now widely accepted view that the media must be extensively and markedly weakened by disease for any dissection to be possible.

The analysis of variance for the entire series of observations is shown in table II.

TABLE II

*Analysis of variance of dissecting pressures*

	Degrees of freedom	Sums of squares	Mean square	Variance ratio F
Between regions . . . . .	2	835891	417946	29.39*
(Linear) . . . . .	1	794449	794449	55.94*
(Second degree) . . . . .	1	41442	41442	2.92
Between cases . . . . .	41	1890284	46104	3.24*
(Between sexes) . . . . .	1	16218	16218	1.14
(Within sexes) . . . . .	40	1874066	46852	3.30*
Interaction . . . . .	82	1165820	14217	1.000
(Region $\times$ sex) . . . . .	2	29696	14848	1.04
(Region $\times$ case) . . . . .	80	1136124	14202	1.00
Within subclasses	252	2625150	10417	0.73
Total . . . . .	377	6517145		

\* = an F value greater than the 1 per cent. level.

The proper comparison of mean squares is, for the preliminary analysis, with the mean square 14217 of the general interaction, and for the more detailed analysis with the mean square 14202 of the interaction "region  $\times$  case". The values of the variance ratio *F* for both "regions" and "cases" are well beyond the 1 per cent. point.

The "between cases" sum of squares has been further analysed to throw light on the influence of sex. The "between sexes" mean square 16218, however, with its corresponding *F* of 1.14, indicates that there are in this material no significant sex differences in dissecting pressure. The significant portion of the "between cases" sum of squares is thus the value 1874066 for "between cases within sexes". This has, however, no special biological meaning, beyond indicating the variability of resistance to dissection from case to case.

The large and significant "between regions" *F* value is important, as it indicates that there is a real regional variation in resistance to dissection. Further, use of the *t* test showed that the resistance of the ascending portion of the vessel is significantly greater than that of the arch, and the resistance of the latter greater than that of the descending portion.

The nature of the trend along the vessel of variation in resistance to dissection may be further analysed by separating the "between regions" sum of squares 835891, based on 2 degrees of freedom, into 2 independent portions, each based on 1 degree of freedom, of which one (say "L") represents the sum of squares accounted for by a linear decrease in resistance down the vessel, and the other (say "S") represents the sum of squares due to any departure from linearity.

If the sum of the 3 sets of observations, each of 126 readings, made on the ascending aorta, the arch and the descending aorta be denoted respectively by A, B, and C, then, as the readings B (for the arch) were made in a region approximately one-third of the distance along the aorta between the regions in which A and C were taken, the appropriate separation of the sum of squares is given by these multipliers:

	For L	For S
A	+4	+2
B	+1	-3
C	-5	+1
Sum of squares of multipliers . .	42	14

In other words the two contributions are :—

$$L = \frac{(4A + B - 5C)^2}{42 \times 126},$$

$$= 794449$$

and

$$S = \frac{(2A - 3B + C)^2}{14 \times 126},$$

$$= 41442.$$

( $L + S = D$ , where  $D$  is the total "between regions" sum of squares, *i.e.* 835891.)

Comparison of  $L$  and  $S$ , each based on one degree of freedom, with the mean square 14202 of the "region  $\times$  case" interaction, furnishes a highly significant value of  $F$  for  $L$ , but not for  $S$ . The evidence therefore favours the view that there is a linear decrease in resistance on passing down the vessel, without any superimposed curvilinear trend.

The importance of using the mean square 14202 of the "region  $\times$  case" interaction for testing the various other mean squares is confirmed by testing this interaction against the "within subclasses" mean square 10417. This procedure gives a value of  $F$  of 1 : 36, with degrees of freedom 80 and 252. The corresponding value of  $z$  is 0.1540, which is just greater than the 5 per cent. point 0.1432.

The possibility that post-mortem changes had altered the resistance to dissection of the media was tested by correlating resistance with the time that had elapsed since death. The figure used to express the resistance of each vessel for this comparison was the mean of the 9 observations made on the vessel. On general grounds it might be expected that post-mortem changes would cause a decrease in resistance; in fact, under the conditions of the investigation (all cadavers being stored in a refrigerator after death), no correlation between the two factors was demonstrated (table III).

TABLE III

*Table summarising correlation analyses*

	Mean dissecting pressure (mm. Hg) correlated with			
	age (years)	hours since death	heart weight (g.)	state of left ventricle*
Correlation coefficient $r$ . . .	-0.3398	-0.054	-0.095	-0.116
Corresponding value of $z$ . . .	0.354	0.054	0.095	0.1165
Standard error of $z$ . . . .	0.1601	0.1601	0.1601	0.1601
$z/S.Ez$ . . . . .	2.21	0.33	0.59	0.73

\* The arbitrary scores 1, 2, 3 and 4 are allotted to the symbols (-),  $N$ , + and ++ for this correlation.

The association with the age of the subject was tested in a similar manner (table III). The expected result of a negative correlation between resistance and age was realised, but although the coefficient attained a significant level it was not large. Clinically, however, the predominance of cases of dissecting aneurysm of the aorta in older persons is striking (Shennan). The present figures suggest that although there is a real diminution in resistance with advancing

years, in normal persons it is of slight degree, and that even in old age the media is a very strong structure.

No correlation was present between the mean resistance to dissection and the gross heart weight as recorded at autopsy, nor was any correlation found between the resistance and the state of the left ventricle when the arbitrary values 1, 2, 3 and 4 were assigned to the symbols (—), N, + and ++ by which the condition of that chamber was recorded (table III). It might be expected that the resistance would decrease with increasing heart weight, the latter being an index to possible arterial degeneration. From this point of view, and also because hypertrophy of the left ventricle is a common finding *post mortem* in cases of dissecting aneurysm of the aorta (Shennan), an evaluation of the correlation between the heart weight and the resistance to dissection in cases in which definite arterial hypertension had been present during life would be instructive if it were possible to delimit accurately the affected (hypertensive) group of subjects. But the absence of a blood pressure record from some of the protocols and the fact that several subjects were admitted to hospital *in extremis* or in a state of shock made this comparison impossible.

The association between the degree of atheroma present in the various regions of the vessel and the corresponding resistance to medial dissection in those regions was examined by an analysis of

TABLE IV  
*Co-variance of amount of atheroma and mean dissecting pressure*

		Sums of squares and products			Reduced values			
	Degrees of freedom	Atheroma $\Sigma x^2$	$\Sigma xy$	Pressure $\Sigma y^2$	Degrees of freedom	Reduced $\Sigma y^2$	Mean square	F
Regions	2	30.9683	—5082.660	835891				
Cases	41	42.8016	—1316.098	1890284				
Interaction (error)	82	15.0317	— 527.419	1165820	81	1147314	14164	
Total	125	88.8016	—6926.177	3891995				
Regions + error	84	46.0000	—5610.079	2001711	83	1317515		
Cases + error	123	57.8333	—1843.517	3056104	122	2097339		
Difference for testing "regions"					2	170201	85101	6.01*
Difference for testing "cases"					41	1850025	45122	3.18*

\* = an F value greater than the 1 per cent. level.

covariance in which the arbitrary values 0, 1, 2 and 3 were assigned to the symbols O, M, + and ++ by which the degree of severity of the atheroma was originally recorded. The results are set out in table IV.

The error term of table IV yields a value of 34.47 (with S.E. 30.74) for the regression of dissecting pressure on degree of atheroma—not a

significant value. Thus there is no association between dissecting pressure and severity of atheroma. A causal relationship between these two variables was not anticipated. Atheroma, being an essentially patchy condition, could hardly be expected to influence the strength of the media in regions other than those immediately underlying the patches.

### Summary

Three regions of the aorta of each of 42 adult cadavers were examined by a technique which determined the pressure required to produce experimental dissection and splitting of the planes of the tunica media. The results of an analysis of the findings may be summarised as follows :—

1. The pressure required to produce medial dissection is almost invariably far in excess of the aortic blood pressure, even in severe hypertension. This indicates the virtual impossibility of producing a dissecting aneurysm by the access of blood during life, through an intimal defect, to a media of normal strength.

2. A definite linear gradient of decreasing resistance to dissection by the media was demonstrated on passing down the aorta, the ascending portion being significantly stronger than the arch, and the arch than the descending portion.

3. A weak negative correlation was found to exist between the resistance to dissection and the age of the subject.

4. No sex differences in powers of resistance were present; the degree of associated atheroma and the state of the left ventricle were not correlated with the resistance of the media; and in the refrigerated cadavers used in this investigation there was no association, either positive or negative, between the strength of the media and the number of hours elapsing since the death of the subject.

We wish to express our indebtedness to Mr Ian Sutherland, of the Institute of Social Medicine, Oxford, for advice on the analyses of variance and covariance; and to Mr G. Ferguson for technical assistance.

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576 . 8 . 094 . 1 (*Bacterium friedländeri*): 616 . 36 : 616 . 155 . 394

## MASSIVE COLONY FORMATION OF *BACTERIUM FRIEDLÄNDERI* IN THE LIVER IN AGRANULOCYTOSIS

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(PLATES IX AND X)

THE usual cause of death in agranulocytosis is bacterial invasion of a body which has been stripped of its leucocyte defences (Dameshek and Wolfson, 1942; Nixon, Eckert and Holmes, 1943). The invading organisms may be any of the ordinary flora of the mouth and upper respiratory passages, and they lead to local ulceration and generalised septicæmia, usually without features of sufficient bacteriological interest to cause them to receive much attention in the literature. The case now reported is, however, of both bacteriological and pathological interest in that certain organs of the patient became, during life, a veritable culture medium in which isolated large colonies of bacteria developed, so large that they presented a considerable diagnostic problem at autopsy. The true nature of the lesions was finally established only by histology.

### CASE REPORT

#### *Clinical history*

The patient, a woman aged 47, became ill at the beginning of January 1947 with an "influenzal cold", the main symptoms of which were fever and epistaxis lasting 5 days. Hæmaturia and deafness developed at the same time and continued for the rest of the illness. On 15th January purpuric spots appeared on the left shoulder, and severe bleeding occurred from the lower gums (the upper jaw was edentulous). The gums became very painful and some cough developed. She was admitted to hospital on 5th February in a very weak condition, with a temperature of 105° F. The lower gums were dark red, swollen and ulcerated, with adherent blood clot. The upper gums were normal but there were large hæmorrhages into the faucial pillars. There were numerous petechiæ over the body and limbs, with large ecchymoses over both tibiæ. Hæmic murmurs were present over the heart. The patient was too ill for satisfactory examination of the lungs.

Hess's capillary permeability test was positive. Blood examination showed hemoglobin 52 per cent., R.B.C. 2,340,000 and W.B.C. 600 per c.mm. No platelets were seen in films. A differential count was made only with great difficulty owing to the paucity of cells; a count of 20 white corpuscles gave

18 lymphocytes, 1 polymorph and 1 monocyte, with no immature cells. Sternal puncture showed a hypocellular marrow, with a differential count on 300 cells as follows:—

Erythroblasts and normoblasts . . . . .	3.3 per cent.
Myelocytes and metamyelocytes (half of them abnormal) . . . . .	8.0 „
Neutrophil leucocytes . . . . .	1.0 „
Eosinophil myelocytes and leucocytes . . . . .	1.0 „
Lymphocytes . . . . .	84.0 „
Plasma cells . . . . .	1.0 „
Monocytes . . . . .	1.7 „

A diagnosis of aplastic anemia was made, with emphasis on the granulocytopenic aspect. The patient was given pentnucleotide and several blood transfusions but did not respond. Her pyrexia continued at 104-105° F., and she went rapidly downhill and died on 13th February. A blood count shortly before death gave 400 W.B.C. per c.mm., with 98 per cent. lymphocytes.

### *Post-mortem examination*

The autopsy was performed 4 hours after death during a time of severe frost with no heating in the mortuary. The body was extremely pale. There were numerous petechiæ and large ecchymoses in the skin of the trunk and limbs. The lower gums were dark purple in colour and covered with clots of blood. Some dried blood was present in the left external auditory meatus.

*The fauces, base of tongue and laryngeal orifice* showed the usual lesions of agranulocytic angina. They were deep purple from submucosal hæmorrhages, and showed multiple circular ulcers which varied from 2 to 5 mm. in diameter and had necrotic bases with no induration and little surrounding œdema.

*The lungs* were extensively consolidated, with involvement of all but the upper part of the upper lobes. The consolidated portions were dark red in colour as a result of hæmorrhage, with a few areas of lighter brick red or brown where necrosis was present. In addition there was much fluid in the lung tissue. The hilar lymph glands were dark red and wet on section. The larger bronchi and the trachea contained tenaceous blood-tinged mucus. The visceral pleura over both lower lobes showed a scanty fibrinous exudate which was stained brown by blood pigment, and there was blood-stained fluid in the right pleural cavity.

*The liver* presented a remarkable appearance. It was pale and rather fatty, with the lobular pattern distinctly outlined. Scattered throughout it there were a number of circular white areas, usually about 5 mm. in diameter. These had a firm, white, opaque appearance, and were sometimes but not always surrounded by a very thin margin of congestion and hæmorrhage (fig. 1). They were present both in the substance of the liver and on the surface, where they projected as low discs. Their nature was puzzling, since they had neither the appearance nor the consistence of abscesses, and the zone of surrounding congestion

*BACT. FRIEDLÄNDERI* INFECTION IN AGRANULOCYTOSIS



FIG. 1.—Cut surface of liver showing white opaque nodules (natural size).

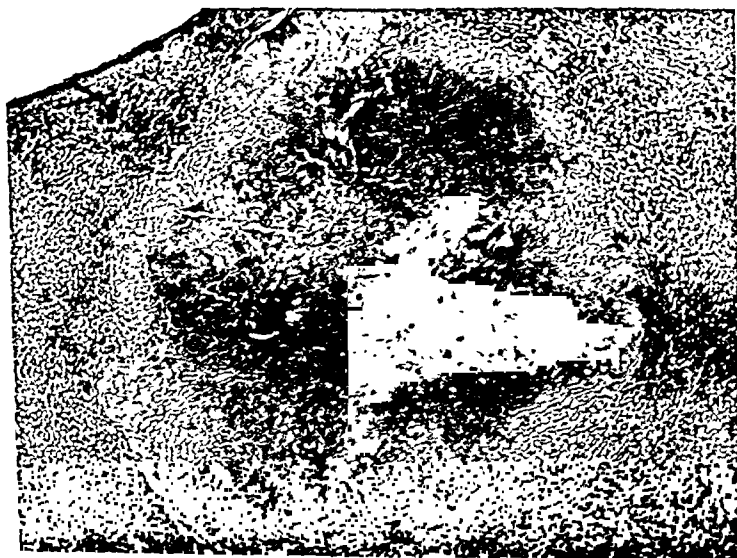


FIG. 2.—Liver showing a "colony" of organisms without surrounding capsule or cellular reaction.  $\times 30$ .



was difficult to associate with tumour-formation, either simple or malignant. A careful but unsuccessful search was made for any primary growth. In view of the failure to establish the nature of these masses by naked eye inspection, the diagnosis was left until after histological examination.

*The other lesions* in the body were mainly hæmorrhagic. The kidneys were nearly white; the subcapsular surface was studded with petechiæ, while the cut surface showed a well-marked zone of hæmorrhage outlining the junction of cortex and pyramids. There was a small recent infarct at the lower pole of the right kidney. The suprarenal glands and the spleen showed numerous petechiæ. There were numerous non-ulcerated submucous hæmorrhages in the stomach and to a lesser extent in the duodenum, lower ileum and colon. Hæmorrhages were present between the layers of the mesentery, under the epicardium and endocardium and into the pia-arachnoid over the vertex of the brain, with smaller petechiæ in the internal capsule and cerebellum. The vertebral bone marrow was red. The femoral marrow was mainly fatty but a few foci of hæmopoiesis were present in the neck and upper part of the shaft. There were a number of hæmorrhages into the fatty marrow.

### *Histology*

Microscopically the liver lesions present a very peculiar appearance. The nodule shown in fig. 2 consists of a mass of fairly homogeneous basophil material filling the spaces between the liver columns. Near the margin this material has compressed the liver columns into thin atrophic strands, and the atrophy increases in severity towards the centre, where the liver cells have completely disappeared so that only the basophil material can be seen. In general the distribution of the material suggests that of intense local amyloid infiltration but the appearances and staining reactions are rather those of mucin. The margin of the lesion is abrupt but there is no capsule or cellular reaction of any kind. Some of the larger lesions show scanty infiltration of the margins by mononuclear cells which contain mucoid material in the form of dark blue threads of mucus with small round spaces intervening. The entire lesion at this low magnification gives the impression of a rather odd type of mucoid carcinoma in which all the tumour cells have been "drowned" in their own secretion.

Higher magnification reveals that the basophil material consists of very numerous small rod-shaped organisms suspended more or less uniformly in a mucoid substance (fig. 3). These bacilli are Gram-negative and have the morphological appearances of encapsulated *Bact. friedländeri*. Unfortunately no cultures had been made from the fresh material so that it is impossible to establish the identity of the organism, but the microscopic appearances of the bacilli with their thick mucoid capsules are very characteristic. In the larger

and presumably older lesions the bacilli in the centre stain poorly and in some places have disappeared.

The lung lesions are basically similar to those in the liver. The consolidated areas vary in appearance in different parts. Sometimes the alveoli are packed with red blood corpuscles to the exclusion of other cells; sometimes they contain a fibrinous exudate with only occasional lymphocytes or mononuclear cells. Over large areas of the lung, however, they are distended with the same basophil material as is seen in the liver, and contain the same bacilli, though usually with less well-marked capsules (fig. 4). In a number of alveoli there is a more serous type of exudate in which the bacilli appeared to be multiplying.

Sections of most of the other organs (lymph nodes, bone marrow, suprarenals, stomach and kidneys) reveal many capillaries blocked for long distances by these organisms, usually with well-marked mucoid capsules. No reaction to these "growing emboli" is apparent.

#### COMMENT

The finding of large colonies of bacteria in the liver at autopsy at once raises the question whether these might have grown in the body after death. For the following reasons it would appear that this explanation is incorrect.

(a) The colonies in the liver are up to 5 mm. in diameter, and the patient had been dead for only 4 hours. Even if the liver were a perfect culture medium, it would be difficult to obtain so much growth in so short a time. In addition, the larger lesions show the bacilli to be degenerating, an indication that they had been there for some time.

(b) The histological appearances of the liver columns compressed between the bacterial masses are those of slow pressure atrophy of live liver cells and not of post-mortem crushing.

(c) There is congestion around the nodules and an occasional attempt at cellular defence in the form of a few mononuclear cells. The absence of polymorphs around the nodules is adequately explained by the agranulocytosis.

The conclusion seems inescapable that the nodules in the liver developed during the life of the patient. It is much more difficult to form any estimate of the duration of these nodules before the patient died. From the histological degree of atrophy of liver-cell columns and in particular the complete disappearance of liver cells from the centre of the lesions, it would appear that the nodules must have been growing for a few days at least. On the other hand the small masses of bacilli growing in the capillaries could well have been there for a much shorter time and were possibly of pre-agonal growth.

The two points of interest in this case are :—

(1) *Bact. friedländeri* grew apparently unhindered in the tissues of a patient who had virtually no polymorphonuclear leucocytes.

*BACT. FRIEDLÄNDERI* INFECTION IN AGRANULOCYTOSIS

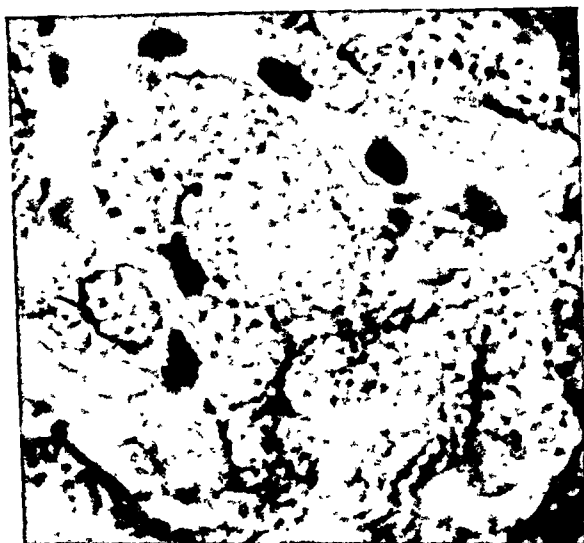


FIG. 3.—Liver. A field from near periphery of lesion in fig. 2. Capsulated bacilli in sinusoids between atrophic liver columns.  $\times 1200$ .

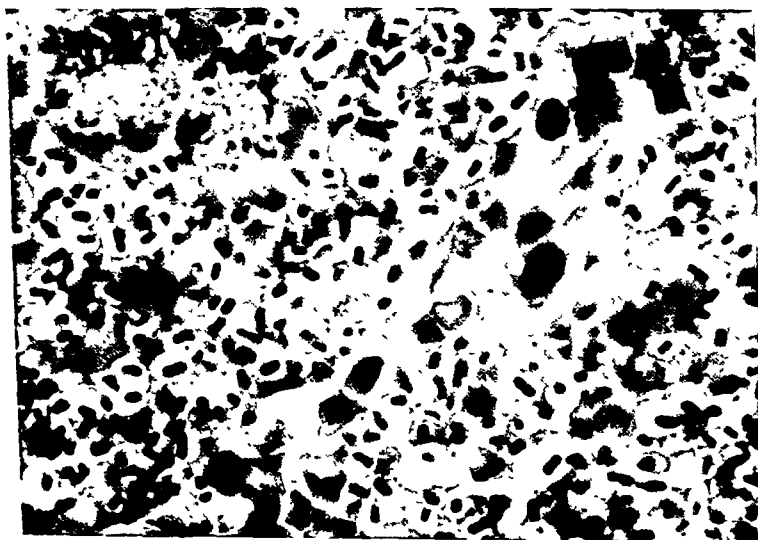


FIG. 4.—Lung showing alveolar "exudate" consisting almost entirely of bacilli.  $\times 1200$ .





There appears to have been no humoral resistance of any kind: in fact the tissue fluids seem to have acted as a culture medium for the organism. It is impossible to say whether the body normally lacks true humoral resistance to *Bact. friedländeri*, or whether any humoral resistance normally present is produced by polymorphs, or again whether the entire resistance of the body to *Bact. friedländeri* is strictly a cellular one according to Metchnikoff's original conception. Presumably the final overgrowth of bacilli in the lungs, in what might otherwise have been an ordinary *Bact. friedländeri* pneumonia, was due to the same factors.

(2) The organisms in the present case do not appear to have produced any locally acting toxins: the liver columns in the neighbourhood show simple pressure atrophy without necrosis of the parenchymal cells. This raises the question whether the final necrosis of tissue seen in *Bact. friedländeri* infection unaccompanied by agranulocytosis is due, not to the presence of the bacilli, but to the presence of polymorphonuclear leucocytes.

Swedin and Liljestrand (1945) report the occurrence of splenic and hepatic "abscesses" due to *Bact. friedländeri* infection in a patient with agranulocytosis. Their patient, a woman of 60, after a prolonged illness, always with a low white-cell count, developed almost complete agranulocytosis during the last ten weeks of life. During this period the polymorphonuclears numbered only 60-150 per c.mm. At autopsy the liver and spleen showed numerous yellowish-white foci, the largest being the size of a hazel-nut. In many cases these foci were surrounded by a zone of hæmorrhage. Microscopically the centre of the lesions showed "marked necrosis", with a peripheral zone of bacterial masses and some karyorrhectic leucocytes. In some cases these foci were surrounded by granulation tissue infiltrated by round cells. The paper is not illustrated by photomicrographs and it is impossible to say whether the word "necrosis" was used with the specific meaning of death of the liver cells, or whether it was applied as a description of the somewhat structureless centre of the lesion from which the tissue cells had disappeared.

*Bact. friedländeri* is not, according to the literature, a frequent terminal invader in agranulocytosis, and Swedin and Liljestrand's case is the only one I have found in which anything like comparable lesions have been described.

These two cases suggest that the possibility of such colony formation during life should be borne in mind in making autopsies on patients who have died with agranulocytosis, in order that the bacteria responsible may be identified by culture.

#### SUMMARY

Large colonies (5 mm. in diameter) of organisms with the morphological characters of *Bact. friedländeri* were found in the liver of a

patient who died of agranulocytosis and aplastic anæmia. The organisms had apparently grown without any resistance, humoral or cellular, on the part of the body. The patient had also a hæmorrhagic pneumonia due to the same organism.

I wish to thank Professor Sheehan and Professor Downie for help and advice, Dr Coope for access to the clinical notes, and Mr Beckwith for the photography.

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# THROMBOSIS AS A FACTOR IN THE PATHO- GENESIS OF AORTIC ATHEROSCLEROSIS

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(PLATES XI-XVI)

It has been shown (Duguid, 1946) that thrombi in the coronary arteries become organised and form fibrous thickenings which are indistinguishable from atherosclerosis. The same occurs in the aorta, where mural thrombi are commoner than is generally supposed. In the aorta such thrombi comprise not only the familiar masses of fibrin which form in relation to atherosclerotic ulcers, but also a variety of fine fibrinous encrustations many of which are too small to be seen by the naked eye. Although these encrustations occur with considerable frequency, even in young subjects, they are a form of lesion which does not appear to have been taken into account in the pathology of aortic disease. Nevertheless they become organised and are undoubtedly a source of intimal thickening.

## *The larger mural thrombi*

The transformation of mural thrombi is essentially the same in the aorta as in the coronary arteries, but the appearances produced are somewhat different and there are certain features which call for special description. These are best shown in some of the larger examples (figs. 1-7).<sup>\*</sup> Most aortic thrombi are composed mainly of fibrin, which when newly formed is loose in texture and somewhat ragged (fig. 1), but which later becomes more compact. At first the fibrin is exposed to the blood stream, but soon a layer of endothelium grows over its surface (fig. 2), and this is followed by the formation of a layer of fibrous tissue in the sub-endothelial zone (fig. 3), making the thrombus appear as if it were a part of the intima. Two forms of change then follow, both of them tending to accentuate this effect. The first consists of a condensation of the fibrin whereby it comes to resemble fibrous tissue ; the second is a true organisation.

<sup>\*</sup> All illustrations are taken from frozen sections which, it should be emphasised, are essential in a study of lesions in the larger arteries. The dehydration and shrinkage involved in paraffin embedding tends to alter the non-cellular tissues to such an extent that in paraffin sections fibrin may be almost indistinguishable from fibrous connective tissue.

The first change is best illustrated in parts where, as frequently happens, there has been recurring thrombosis with one deposit on top of another (fig. 4). Whereas the newly formed fibrin appears reticular or somewhat flocculent, older deposits are compact and almost homogeneous, but often with a laminated structure which in section gives a coarse fibrous appearance. In them the fibrin has lost its specific staining properties and takes a variable yellow with picro-fuchsin, so that in paraffin sections it has the appearance of hyaline fibrous tissue. Thus before organisation is far advanced many of the older deposits no longer look like mural thrombi and may be mistaken for degenerate connective tissue.

Organisation is a slower process and varies with the size of the thrombus. Some of the larger are penetrated by capillaries and go through the same process as an organising venous thrombus or fibrinous exudate (fig. 5), and it is perhaps this which accounts for the apparent vascularisation of some atherosclerotic plaques. The majority of smaller thrombi, on the other hand, are transformed by an avascular process in which cells alone are the active agents. Cells from the subjacent intima penetrate the fibrin and form centres around which it is transformed into collagenous substance (fig. 4). Thus an advancing zone of organisation is formed which gradually overruns the thrombus and converts it into fibrous tissue. There are however factors which complicate this picture. In the aorta thrombosis tends to recur, with the formation of multiple deposits as seen in figs. 3 and 4, and the appearances produced are varied, depending not only on the size of the deposits but also on the time which elapses between their formation. When one follows quickly on the other the new layer of fibrin lies directly on the old, as in fig. 4, but when a sufficient interval elapses, the older deposit may be covered with a layer of fibrous tissue before the next is laid down, and thus alternating strata of fibrin and fibrous tissue are formed, as in fig. 3. With repeated thrombosis and organisation thick accumulations of fibrous tissue may be produced, but not infrequently there may be found, deeply embedded within them, layers of hyaline or "fibrinoid" substance (fig. 6), representing incompletely organised thrombi, with nothing in their appearance to show that they originated as surface deposits. These remnants sometimes undergo fatty changes and sometimes softening, thus forming atheromatous foci.

### *The finer fibrinous encrustations*

The larger thrombi described above are by no means the commonest form of mural deposit, and one cannot go far in the study of aortic thrombosis without being impressed by the frequency with which thin layers of fibrin are to be found on the intimal surface. These are most often seen in association with the larger thrombi (fig. 7), but are not always confined to them, for they sometimes occur in the

## AORTIC ATHEROSCLEROSIS



FIG. 1.—Recent mural thrombus forming a thick deposit towards the left and a thin encrustation towards the right. Sudan III and hæmalum.  $\times 110$ .

FIG. 2.—Somewhat older thrombus than that shown in fig. 1, with a layer of endothelium on its surface. Sudan III and hæmalum.  $\times 110$ .



FIG. 3.—Mural thrombi of different ages. Towards the left there is a thick mass of condensed, almost homogeneous fibrin, representing a mural thrombus of some standing. It is covered by endothelium and a thin layer of fibrous tissue, while on its surface at the extreme left there are flakes of more recent fibrin encrustation. Beneath the thick mass and towards the right of the picture there is an older fibrinous deposit showing ill defined lamination. It also is covered by a layer of fibrous tissue, so that it is separated from the overlying thrombus. Its basal layers are invaded by connective tissue cells. Sudan III and hæmalum.  $\times 80$ .



## AORTIC ATHEROSCLEROSIS

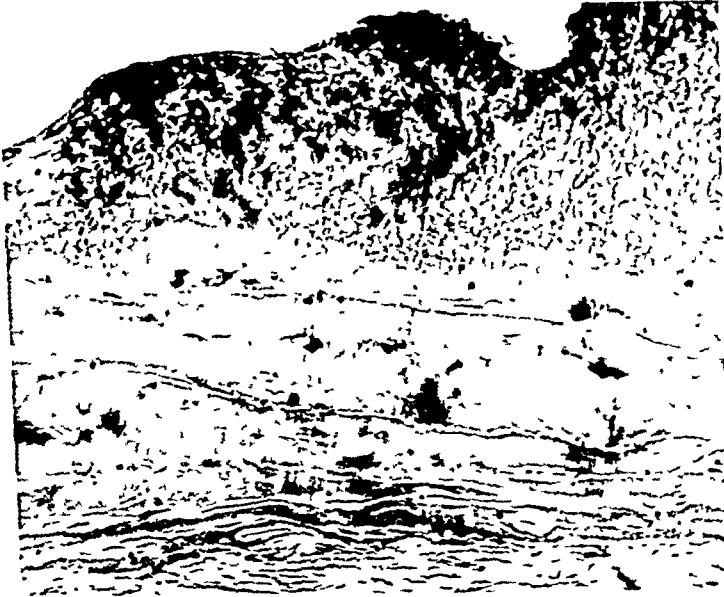


FIG. 4.—Recurring mural thrombosis with early organisation. Two strata of fibrin of different ages are seen, with a layer of endothelium partly covering the superficial one, but with no fibrous layer between. The more recent deposit is granular, the older dense and laminated. The basal layers of the older deposit show early organisation, with connective tissue cells invading them and forming centres of collagen formation. Sudan III and hæmalum.  $\times 130$

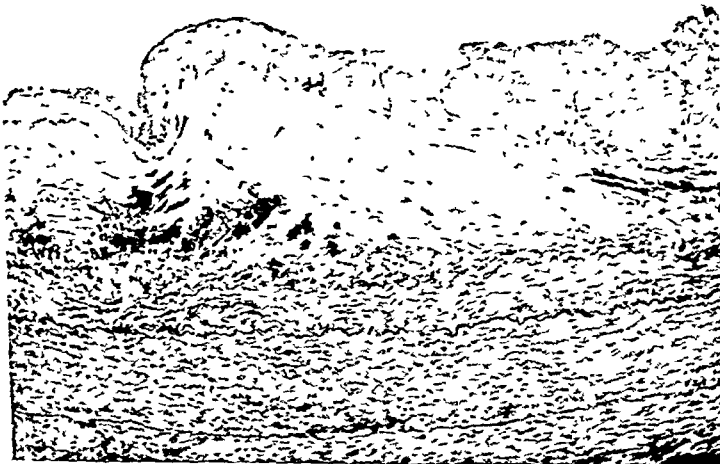


FIG. 5.—Large mural thrombus showing early vascularisation by scattered capillaries. Sudan III and hæmalum.  $\times 60$ .





## AORTIC ATHEROSCLEROSIS

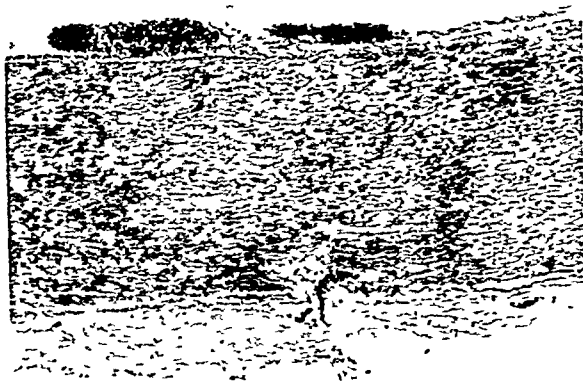


FIG. 6.—An old, incompletely organised mural thrombus lies a little below the surface. It consists of "fibrinoid" material embedded in a fibrous thickening of the intima. In the overlying fibrous tissue there is a darker zone close to the surface, representing a more recent deposit not fully organised but showing hyaline change. In the deeper layers of the intima the black patches are hyaline material with fatty change. Sudan III and hæmalum.  $\times 120$ .



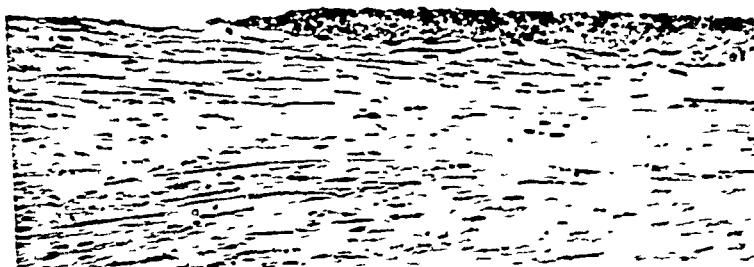
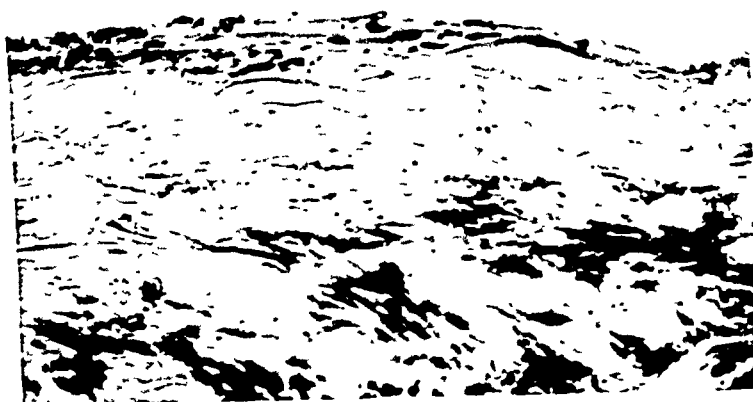
FIG. 7.—Large fibrinous deposit of some standing, with recent encrustation on its surface. Sudan III and hæmalum.  $\times 40$ .

FIG. 8.—Fine encrustation in the aorta from a child aged 3 who died from lymphatic leukæmia. It is packed with leukæmic cells. Hæmalum.  $\times 70$ .





AORTIC ATHEROSCLEROSIS



FIGS. 9 and 10.—Varieties of fine encrustations. Sudan III and hæmalum.  
× 150 and 120.

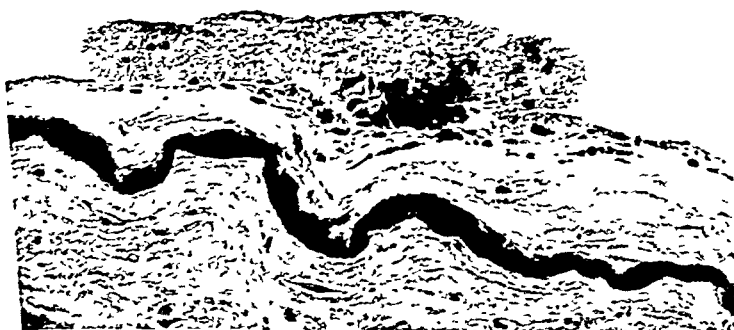


FIG. 11.—Minute fibrinous flake with endothelial cells clumped beneath it. The dark streak in the deeper layers of the intima is a layer of hyaline substance with fatty change. Sudan III and hæmalum. × 200.



apparently healthy aortas of quite young children (fig. 8). They are very variable and may consist of no more than a few ragged strands of fibrin and leucocytes, so that they look like mere frayings of the surface (fig. 9). Sometimes they are extensive (fig. 10), and sometimes localised, forming minute flakes (fig. 11). Occasionally they may be seen by the naked eye or with the aid of a hand lens as slight roughenings of the surface, but more often they are microscopic. In the young aorta they are most commonly to be found near the mouths of branches (fig. 12), where superficial fatty streaking occurs, and they are not infrequently associated with this condition.

As a rule these fine encrustations appear to be of recent origin and may at first be taken for post-mortem clots, but the firmness with which they adhere to the vessel wall (fig. 16) and withstand the rough manipulations involved in the cutting and staining of frozen sections makes it evident that they are present during life. This is confirmed by the fact that in some of them signs of transformation are recognisable, for, like the larger thrombi, they also become covered with endothelium (fig. 14) and, passing through the same process of condensation (fig. 15) and hyaline change (fig. 16), are ultimately changed into fibrous thickening.

#### *The incidence of mural thrombi in the aorta*

If fibrinous deposits are a source of intimal thickening, their importance in aortic disease must depend on their frequency, and it is therefore desirable to obtain some measure of this. To estimate the frequency of lesions, many of which are microscopic, in a large vessel like the aorta is difficult, and in this investigation it was decided to obtain a rough measure of their incidence by seeing how frequently mural deposits could be found in a search limited to one histological section in each case. The aortas from an unselected series of autopsies were taken, and in each a section was made of the part which, on inspection with a hand lens, looked most likely to be affected. Such a hit or miss method was calculated to give an underestimate, but the findings were surprising.

Out of 50 aortas, fibrinous deposits of one kind or another were found in no less than 19, those in 15 being of the fine type. All of them consisted of recently formed fibrin, the identity of which was beyond question. The age incidence ranged from three to seventy-three years, 4 out of the 8 cases under thirty being positive. One of these, a child aged three, died from lymphatic leukaemia; the other, aged seven, from a cerebral tumour. The deposits were of course most plentiful in the atherosclerotic aortas of old subjects, especially where there was ulceration, but apart from this there was no apparent relationship to any particular disease or to the duration of illness. Thus the survey, restricted though it was, showed that aortic thrombosis is a common occurrence, arising not infrequently in

childhood and tending to increase with advancing years. It seems likely, therefore, that in the adult aorta a considerable amount of intimal thickening must arise from this cause.

### *Discussion*

It remains to be considered what relationship if any the organisation of mural thrombi bears to atherosclerosis. Atherosclerosis is a disease characterised by fibrous thickening of the intima with fatty change. It is clear that mural thrombosis may add to the thickening, but whether it is a factor in the development of the disease or merely a superadded complication is another question. The development of atherosclerotic thickenings, and their relationship to the fatty changes have never been satisfactorily explained. Since the advent of Virchow's cellular theory it has been taken for granted that they represent overgrowths of the intimal connective tissues. Yet few who have studied the lesions can have been wholly satisfied with this hypothesis, since signs of cellular proliferation are hard to find, cells tending to be least numerous where the thickenings are greatest. In earlier years Rokitansky regarded atheroma as the product of fibrinous deposits on the vessel walls and the findings recorded above support this view. Fibrinous deposits may not be the only source of intimal sclerosis but at least they—and their sclerosing effects—can be demonstrated without difficulty.

In considering the possible part played by thrombosis in the development of atherosclerosis, it is necessary to enquire into the relationship of fatty changes to thrombosis. In aortic atherosclerosis the fatty changes are mostly seen in the deeper layers of the intima or in the sub-intimal zone, where any connection with mural deposits would be hard to trace, but they may also occur near the surface, and there a relationship is sometimes apparent. A fine sprinkling of lipid droplets appears in those masses of hyaline substance which represent buried mural thrombi, and would appear to be a product of the thrombi. But this does not hold for all the fat which appears in the vessel wall. In superficial fatty streaking and in the cholesterol sclerosis of rabbits lipid deposition appears as a primary change, presumably the result of disordered fat metabolism, and here it seems possible that a relationship of a different kind may exist. In superficial fatty streaking fat-laden cells tend to collect under the endothelium and to elevate it in such a way as to expose it unnaturally to the force of the blood stream (fig. 17), and it seems likely that rupture may sometimes occur. When it is recalled that the larger mural thrombi are commonly associated with ulceration, which is the result of rupture following atheromatous degeneration of the intima, it will easily be appreciated that the finer encrustations may arise from similar ruptures on a smaller scale. It would be difficult to demonstrate this, but it seems significant that the finer encrustations tend to occur

## AORTIC ATHEROSCLEROSIS



FIG. 12.—Minute fibrinous encrustation at the origin of a branch of the aorta. Otherwise vessel wall appears healthy. Sudan III and hæmalum.  $\times 70$ .



FIG. 13.—Two strata of fibrin encrustation on the surface of an atherosclerotic plaque. Both are condensed and hyaline, and stain more darkly with hæmalum than the underlying fibrous tissue. The more superficial and darker is partly detached at its margin as if tending to peel off the surface. Hæmalum.  $\times 125$ .



FIG. 14.—Fine fibrinous encrustation with a newly formed covering of endothelium. Sudan III and hæmalum.  $\times 110$ .





AORTIC ATHEROSCLEROSIS

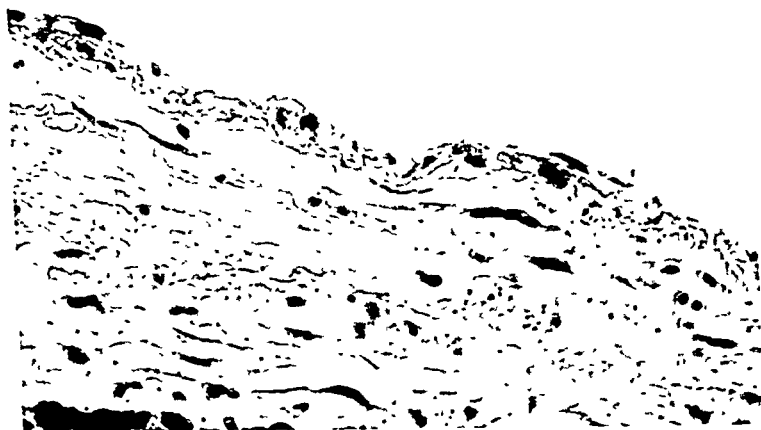


FIG. 15.—Fine fibrinous encrustation covered with endothelium and showing condensation and hyaline change. Sudan III and hæmalum.  $\times 200$ .



FIG. 16.—Minute encrustation showing hyaline transformation. Hæmalum.  $\times 200$ .

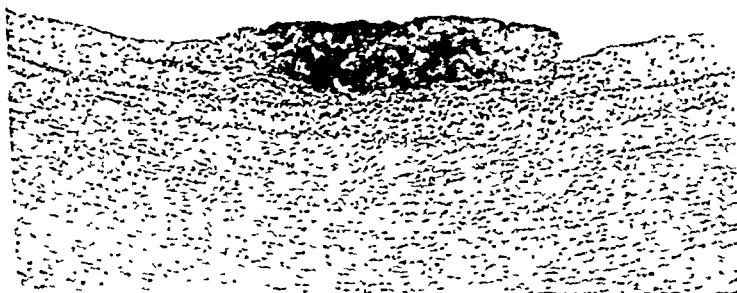


FIG. 17.—Superficial fatty streak in the aorta of a child aged 12, killed in an accident. A cluster of fat-laden cells (unstained) in the sub-endothelial zone forms a protrusion of the surface. No fibrinous encrustation is present. Hæmalum.  $\times 50$ .



near the origin of branches, where fatty streaking is common, and in fact are often associated with this condition. Thus fatty changes may be both a cause and an effect of thrombosis, and the two may be as it were, reciprocating factors in what amounts to a vicious cycle.

The fine fibrinous encrustations are themselves small, but they must each add something to the thickness of the vessel wall, and if often repeated in the course of a life-time, may ultimately give rise to considerable thickenings. The frequency of their occurrence in post-mortem material suggests that they must be a common source of thickening, and there seems to be no reason to exclude them as a cause of the thickening which constitutes atherosclerosis. In the past our approach to the problem of intimal sclerosis has been dominated by the growth hypothesis and consequently attention has been directed mainly to a search for agents calculated to excite connective tissue proliferation in the arteries. The study of thrombosis opens a fresh approach to the problem, and with it comes the implication that the thickenings may depend not only on factors which act upon the tissues of the vessel wall, but also on those which govern fibrin formation in the circulating blood.

### *Summary*

In the aorta organised mural thrombi are a common cause of intimal thickening, but the process of organisation tends so to alter their appearance that they are no longer easily identifiable as thrombi. Many of them are microscopic and so are liable to be overlooked. They occur at almost any age and tend to recur, thus causing progressive thickening. They sometimes undergo fatty change, producing an appearance identical with atherosclerosis, and the possibility that they are a factor in the development of that disease seems more than likely.

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## THE NATURE OF ANÆMIA IN LEUKÆMIA

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(PLATES XVII AND XVIII)

THE purpose of this paper is to describe some cases of unusual anæmia occurring in leukæmia and to consider the nature of the disturbance of red-cell formation in this disease.

Fifty consecutive new cases of leukæmia attending the General Infirmary at Leeds in the years 1945, 1946 and part of 1947 were referred to the department of pathology for blood examination. Table I shows the incidence and severity of the anæmia in these

TABLE I

*Incidence and severity of anæmia in 50 untreated cases of leukæmia at time of first diagnosis*

		Acute leukæmia (14 cases)	Chronic lymphatic leukæmia (16 cases)	Chronic myelogenous leukæmia (20 cases)
Hæmoglobin (g. per 100 ml.)	16-18	0	1	3
	14-16	0	2	1
	12-14	0	1	3
	10-12	4	2	2
	8-10	1	2	4
	6-8	3	2	4
	4-6	6	3	2
	2-4	0	3	1
Erythroblasts present in first blood film		10	2	16
Range of W.B.C. per c.mm.		1200 to 69,600	1000 to 422,000	1800 to 420,000
Sex distribution . . .		7 M : 7 F	13 M : 3 F	12 M : 8 F
Age range (years) . . .		4-72	8-64	23-63
Age average (years) . . .		36	42	46

cases at the time of first diagnosis of leukæmia. Fourteen were cases of acute leukæmia, nearly all of which, so far as we can now determine, were instances of acute myelogenous or myeloblastic leukæmia. Of the remaining 36 cases of chronic leukæmia, 16 were of lymphatic type, 20 of myelogenous. Some aleukæmic cases are included in all three groups. The sex distribution and age range are shown for each group. There was a preponderance of males in the whole series. Ages varied

widely but tended to be lower in acute leukaemia. Importance is attached to the distribution of the haemoglobin content of the blood and to the frequency of finding erythroblasts in the first blood films.

Significant anaemia of less than 12 g. Hb. per 100 ml. or 82 per cent. Hb. (14.5 g. per 100 ml. being equivalent to 100 per cent.) was present in every case of acute leukaemia, in 75 per cent. of cases of chronic lymphatic leukaemia and in 65 per cent. of cases of chronic myelogenous leukaemia. Severe anaemia below 50 per cent. Hb. (less than 7.3 g. per 100 ml.) was found in acute leukaemia in 64 per cent. of cases, in chronic lymphatic leukaemia in 50 per cent. and in chronic myelogenous leukaemia in 30 per cent.

Panton and Valentine (1929) found normal red cells in over half their cases of chronic lymphatic leukaemia but Wintrobe and Hasenbush (1939) state that anaemia is more likely to be present early in this disease than in the myelogenous form. Our figures agree with this latter view. Anaemia was both more frequent and more severe in chronic lymphatic than in chronic myelogenous leukaemia, but in neither was it so constantly present as in acute leukaemia, an observation with which most authors (*c.g.* Wintrobe, 1946, pp. 690 and 698) agree.

Nucleated red cells appeared in the blood commonly in chronic myelogenous leukaemia and acute leukaemia (most cases of which were probably of myeloblastic type), rarely in chronic lymphatic leukaemia. Forkner (1938, p. 67) states that these cells are frequently found in all types of leukaemia but particularly in the chronic myelogenous form. In myelogenous and acute leukaemia the presence or absence of nucleated red cells seemed to be independent of the degree of anaemia. Neither anaemia or erythroblastosis could be correlated either with the age of the patient or with the numerical value of the total white-cell count. There was insufficient evidence from which to draw any conclusion as to the influence of the duration of the disease on these manifestations. The insidious onset of the chronic disease would in any case make this difficult, but Wintrobe (p. 698) has shown that anaemia increases rapidly in the course of acute leukaemia. Most of our patients with chronic leukaemia received radiotherapy after diagnosis and this had a most variable effect on the anaemia. The development of profound anaemia usually accompanied the terminal phase of the chronic disease.

#### CASE REPORTS

**Case I.** The most severe degree of anaemia in our series was found in this patient, a boy aged 10 years. He had been ill for 7 months and noticeable anaemia had developed in the 6 weeks before coming to hospital. No history of blood loss. Spleen enlarged below umbilicus. Most groups of lymph nodes enlarged. Chronic lymphatic leukaemia. W.B.C. 154,000 per c.mm. Haemoglobin 2.0 g. per 100 ml. (14 per cent.). R.B.C., 900,000 per c.mm. Returned home, no follow-up.

**Case II.** Male, aged 64, a gardener. One month's history of anorexia

without pain; dyspnœa and palpitation on exertion. No palpable enlargement of spleen, liver or lymph nodes. Very pale. Small hæmorrhages in optic fundi. Albuminuria. Renal function tests and X-ray examination of long bones and alimentary tract revealed nothing abnormal. *Blood.* Hb. 25 per cent. (3.6 g. per 100 ml.), R.B.C. 1.1 million/c.mm., C.I. 1.13, M.C.V.\* 100  $\mu$ m, M.C.H.C. 33 per cent., W.B.C. 1000 per c.mm. (neutrophils 34 per cent., lymphocytes 65 per cent., monocytes 1 per cent.), normoblasts 1 per 100 W.B.C. *Sternal marrow puncture.* Blasts 1 per cent., granulocytes 1.3 per cent., lymphocytes 94.6 per cent., normoblasts 3.1 per cent. A highly cellular marrow crowded with small lymphocytes. *Diagnosis.* Aleukæmic chronic lymphatic leukæmia. *Progress.* Temporarily relieved by transfusions. Readmitted after 6 weeks with blood picture essentially the same (Hb. 25 per cent., W.B.C. 900 per c.mm.). Died 8 weeks after first attending hospital. *Autopsy.* Enlargement of cervical glands, spleen (476 g.) and liver (2070 g.). Marrow of femur was red throughout its length but still contained some fat. *Histology.* Leukæmic infiltration by small lymphocytes of lymph nodes, spleen, adrenals and gastric mucosa. Diffuse lymphocytic infiltration of femoral marrow with a very few small scattered foci of erythroblasts. No myeloid metaplasia observed in the viscera.

The very severe anæmia in each of these two cases of lymphatic leukæmia was responsible for bringing them to hospital. There was a history of 7 months' ill-health in case I, but it is impossible to guess how long the leukæmic process had been silently developing in each case. Loss of blood by hæmorrhage did not apparently contribute to the anæmia, which seems to have resulted from progressive hypoplasia of the erythron. The only evidence of red-cell regeneration was the finding of a very occasional erythroblast in the blood films of each case. It is notable however that these two patients, both with extremely severe anæmia, were the only ones in the group of 16 lymphatic leukæmias in which erythroblasts were recorded as being present on first examination. Nucleated red cells are liable to appear in the blood film from any form of anæmia of like severity and their presence in these circumstances does not necessarily indicate hyperplastic activity of the erythroid marrow tissue. Nor was this evident in the autopsy material from case II.

Stimulated activity of the erythron, on the other hand, is much more commonly evident in myelogenous leukæmia. Either actual erythræmia or erythroblastosis with moderate anæmia may be the first evidence of this disease.

*Case III* (not included in table I). Male clerk aged 47 in 1931, when he first came under observation with mild anæmia (Hb. 76 per cent.) and slight splenomegaly. Nucleated red cells were first seen in the blood film a few months later and have been present ever since in numbers ranging from 1 to 10 per 100 W.B.C. In 1935 myelocytes were occasionally found, but the total leucocyte count did not reach an abnormal level until 1939. The diagnosis of chronic myelogenous leukæmia was finally established in 1943, when he was first seen

\* M.C.V. = mean corpuscular volume. M.C.H.C. = mean corpuscular hæmoglobin concentration. The classification of the red-cell series in bone marrow is in accordance with Israëls (1939b). The marrow megaloblast is assumed to play no part in normal post-fœtal erythropoiesis. Reference to megaloblasts in the peripheral blood always indicates megaloblasts of the same nature, i.e. megaloblasts of Ehrlich.



by one of us. At this date the total white cell count was 31,000 per c.mm., with 17 per cent. myelocytes, 60 per cent. Hb. and a greatly enlarged spleen. Anæmia of about this degree persisted and in 1944 was shown to be of microcytic type (M.C.V. 67  $\mu$ ). Total leucocytes were never observed to exceed 52,000 per c.mm. Our records cease in 1945 but he was known to be alive 2 years later.

This man (case III) was thought to be suffering from pernicious anæmia and was treated as such for 12 years without conspicuous benefit. The grounds for this diagnosis were somewhat slender, more especially as gastric analysis showed a hyperchlorhydric curve. But the colour index was at first almost consistently above unity and the microcytic anæmia was a much later development. The few myelocytes which were seen for 4 years before the total leucocyte count rose were presumably interpreted as a leukæmoid reaction in pernicious anæmia. This is not infrequent when the anæmia is severe and during response to liver therapy. The distinction between this leukæmoid reaction and myelogenous leukæmia is discussed by Heck and Hall (1939), who point out that immature leucocytes always disappear in pernicious anæmia under the influence of adequate specific treatment, whereas this does not happen in leukæmia. In our view erythroblastosis in association with only moderate anæmia should always bring myelogenous leukæmia to mind where the case is that of an adult in whom icterus or osseous metastases are not obvious. Mention should also be made of a very rare erythroblastosis of adults described by Émile-Weil and Perlés (1939) and Nordenson (1946) in which widespread extra-medullary hæmopoiesis seems to be responsible for the immaturity of a proportion of the circulating red and white cells.

There is more substantial evidence of pernicious anæmia preceding leukæmia in case IV. Case V is an example of a severe megaloblastic macrocytic anæmia immediately preceding acute leukæmia.

**Case IV.** A female aged 28 in 1934, when she complained of sore tongue and weakness and numbness of arms and legs. In 1936 she was recognised to be suffering from mild anæmia; Hb. 70 per cent. (10.5 g. per 100 ml.), R.B.C. 3.4 million per c.mm., W.B.C. 4200 per c.mm. One nucleated red cell per 100 W.B.C. No apparent immaturity of leucocytes. Gastric achlorhydria. After 6 weeks' treatment with liver and iron: Hb. 90 per cent., R.B.C. 3.8 million per c.mm., no erythroblasts. She continued liver injections intermittently but relapsed in 1941, recovered with regular treatment and maintained good health. In Oct. 1946 she attended out-patients, feeling well. Routine blood examination showed Hb. 95 per cent., R.B.C. 4.5 million per c.mm., W.B.C. 5700 per c.mm. Within three months, in Jan. 1947, she reported at hospital stating that slight injuries caused bruising but she was still able to do her housework normally. She was admitted and the findings are summarised below and in table II.

*Clinical examination.* No enlargement of liver, spleen or lymph nodes. Other systems normal. Traumatic purpura but no petechiæ.

*Special tests.* W.R. negative. Renal function normal. X-ray of long bones and gastro-intestinal tract normal. Ascorbic acid saturation test normal. Histamine-fast achlorhydria. Total plasma protein 6.8 g. per 100 ml.

THE ANEMIA OF LEUKAEMIA



FIG. 1.—Case IV. Blood film of 26/2/47. Two myeloblasts (one with monocytoid nucleus), a normoblast and a lymphocyte. Wright's stain.  $\times 900$ .



FIG. 2.—Case IV. Blood film of 26/2/47. Two monocytoid myeloblasts, a polymorph and a normoblast. Peroxidase stain.  $\times 750$ .

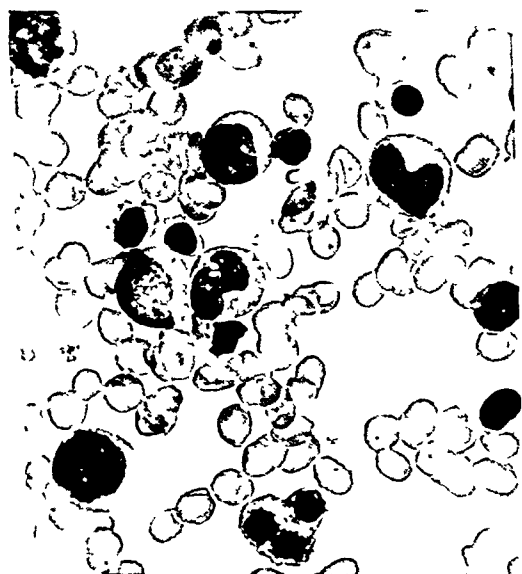


FIG. 3.—Case IV. Sternal marrow of 7/2/47. Normoblasts, myelocytes and giant metamyelocytes. Wright's stain  $\times 600$ .

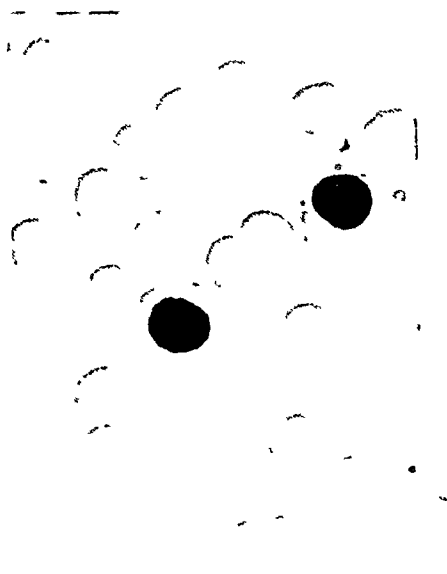


FIG. 4.—Case V. Pre-leukæmic phase. Megaloblasts and macrocytosis in blood film of 30/12/46. Wright's stain.  $\times 1000$ .



*Hæmatology.* Bleeding time indefinitely prolonged. Clotting time and clot retraction normal. Platelets 70,000 per c.mm. Fragility of R.B.C. normal (0.50 to 0.34 per cent. saline). For blood counts see table II.

TABLE II  
Case IV. *Hæmatological findings*

Date 1947	Hb. (per cent.)	R.B.C. 10 <sup>6</sup> /c.mm.	W.B.C. per c.mm.	Myelo-blasts (per cent.)	Myelo-cytes (per cent.)	Meta-myelo-cytes (per cent.)	Poly. (per cent.)	Lymph. (per cent.)	Mono. (per cent.)	Nucleated R.B.C. per 100 W.B.C.
24/1	69	3.07	3900	0	0	0	11	84	5	8
5/2	62	3.0	3500	0	0	0	18	80	2	7
11/2	58	2.6	5900	0	0	0	11	77	12	44
14/2	58	2.6	7000	0	0	0	7	78	15	55
19/2	56	2.4	12,000	0	0	0	10	74	16	50
24/2	40	1.8	21,700	*?	1	2	14	35	48 ?	22
26/2	...	...	24,200	*58	8	7	12	14	1	...

\* See text.

*Sternal marrow biopsy (7/2/47) : differential percentages*

Blasts	3.0	Lymphocytes	8.2	}	10.9
Promyelocytes	0.7	Monocytes	2.7		
Myelocytes N.	24.6	Pro-erythroblasts	0.5	}	37.2
„ E.	0.5	Normoblasts A	4.5		
Metamyelocytes	17.7	„ B	15.7		
Polymorph neutrophils	3.7	„ C	16.5	}	1.4
Megakaryocytes	0.3	Unclassified			
Total nucleated cell count = 96,600 per c.mm.					
Leuco-erythrogenic ratio = 1.2 : 1					

Although a few of the erythroblasts are of large size, their nuclear pattern is not that of megaloblasts. There are many giant metamyelocytes but few mature polymorphs. The myelocytes seem rather deficient in granules. The main features are those of active normoblastic erythropoiesis and some abnormality of granulocyte maturation (fig. 3).

Progressive anæmia and gradually mounting leucocytosis were observed throughout her month in hospital. There was a notable neutropenia and an increasing monocytosis. On 24th Feb. the white cell picture had altered strikingly. Doubt was then felt as to the identity of the cells which were now provisionally classed as monocytes. They had a slaty, almost clear cytoplasm and a nucleus of twisted rope pattern. They proved to be loaded with peroxidase-positive granules although specific-staining granules were scarcely detectable with Wright's stain. Blood films of 26th Feb. are depicted in figs. 1 and 2. On that day the peculiar cells were classified as myeloblasts in accordance with the views of Naegeli (1931). The final blood picture was that of the so-called Naegeli type of monocytic leukæmia (Downey, 1938; Watkins and Hall, 1940) which is, in fact, an acute myelogenous leukæmia characterised by bizarre monocytoid myeloblasts.

*Bone marrow.* Sternal puncture was made on 7th Feb. before leukæmia was manifest and when the diagnosis of pernicious anæmia was alone entertained. The differential marrow-cell count is shown in table II, together with the report issued at that time, as re-examination of the preparations gives us no reason to modify it (fig. 3).

*Clinical course.* While in hospital there was a tendency to bleed from the gums and into the skin. Transfusion was unsuccessful, as rigors developed

within a few minutes of starting drips of compatible blood. Liver extract was injected on 4th Feb. and on 6th Feb. and daily from 14th Feb. The reticulocytes rose from 1.8 per cent. on admission to 7.0 per cent. on the tenth day after the first liver injection. In view of this and of the long period of maintenance liver dosage before admission, the absence of megaloblastosis of the marrow three days after re-starting liver does not exclude the validity of the earlier diagnosis of pernicious anaemia. Both on admission and three weeks later the anaemia was moderately macrocytic (M.C.V. 107  $\mu$ , M.C.H.C. 30 per cent.). The spleen became palpable when leukaemia developed. The patient went home and died there on 4th April. No autopsy was obtained.

**Case V.** Male aged 39, a plasterer, had a short attack of "influenza" in Nov. 1946 with fever and pains in the back and neck. After a few days he resumed work but soon developed septic spots on the scalp and naso-labial folds and became unduly tired. He attended hospital on 28.12.46 and was admitted.

*Clinical examination.* A well-nourished man but strikingly pale. Left naso-labial fold has an indurated lesion about 1 in. across with a dark necrotic centre. Infection of crypts of left tonsil and associated lymphadenopathy. No enlargement of other lymph nodes or of spleen. Albuminuria. Small discrete haemorrhages in both optic fundi.

*Special tests.* W.R. negative. Normal renal function. Normal X-ray appearances of long bones. Histamine-fast achlorhydria. Blood cultures sterile on several occasions while pyrexial.

*Haematology.* Selected blood counts to show progress are included in table III. On admission, severe macrocytic anaemia (M.C.V. 143  $\mu$ , M.C.H.C. 32 per cent.), neutropenia and massive erythroblastosis, with a majority of megaloblasts. Thrombocytopenia and prolonged bleeding time. Manifest leukaemia occurred only four days before death, the total W.B.C. count reaching 37,200 per c.mm. on the day before death. Differentiation of the large mononuclear cells in the final examinations proved difficult, even with the use of peroxidase staining, but the out-pouring of typical myeloblasts and myelocytes established the diagnosis of acute myelogenous leukaemia. Cells of the peripheral blood are illustrated in figs. 4-6.

*Bone marrow.* Sternal marrow puncture was made shortly after admission and showed intense megaloblastic erythropoiesis (fig. 7). Smears were also made from puncture of the sternal marrow immediately after death on 23rd Feb. (fig. 8). The marrow picture had then completely changed to that of myeloblastic leukaemia. On both occasions the marrow smears seemed highly cellular. Differential counts are shown in table III.

*Clinical course.* Liver injections were instituted in view of the severe macrocytic anaemia, the peripheral and marrow megaloblastosis and the gastric achlorhydria. Their effect was seen in the reticulocyte response and in the reversal of the marrow to normoblastic erythropoiesis, though at the same time the marrow had assumed leukaemic characters. Liver was given for 30 days but much blood was lost from repeated epistaxis and the haemoglobin continued to fall, except when temporarily raised by transfusions. Synthetic folic acid only resulted in an insignificant reticulocytosis at the time when the white cell count was beginning to climb. Necrotic lesions of mouth, tongue and fauces developed, and from 4th Jan. fever up to 103° or 105° F. was constant. Penicillin was without effect. The patient succumbed to bronchopneumonia on 23rd Feb. It is interesting to note that leukaemia was suspected on clinical grounds on admission but haematological confirmation was uncertain until shortly before death.

*Autopsy (23/2/47).* Confluent bronchopneumonia of both lower lobes with sanguineous fibrinous pleurisy and pericarditis. Discrete ecchymoses in pleura and intestinal mucosa. Liver 2220 g. Spleen 500 g., with many small recent subcapsular thrombotic infarcts. Greyish-red marrow filled the interior

THE ANÆMIA OF LEUKÆMIA

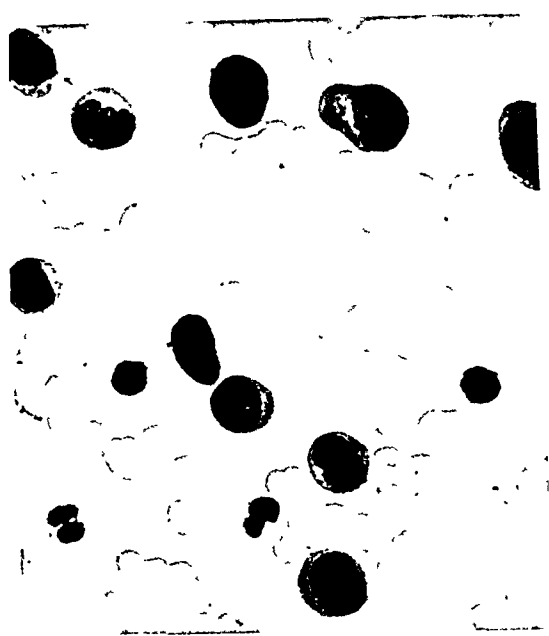


FIG. 5.—Case V. Acute leukæmic phase. Blood film of 20/2/47. Myeloblasts and myelocytes, some with monocytoid nuclei. Wright's stain.  $\times 600$ .



FIG. 6.—Case V. Blood film of 20/2/47. A neutrophil polymorph, two granular myelocytes and two less differentiated myeloblasts. Wright's stain.  $\times 1800$ .



FIG. 7.—Case V. Sternal marrow of 3/1/47. Pre-leukæmic phase. The cells are all megaloblasts with the exception of a "smudged" neutrophil at the top and two myelocytes. Wright's stain.  $\times 900$ .



FIG. 8.—Case V. Sternal marrow shortly after death on 23/2/47. Myeloblasts and myelocytes now predominate. Some are of monocytoid form and nucleoli are prominent in others. Peroxidase staining showed 62.5 per cent. of marrow cells bearing positive granules. Wright's stain.  $\times 500$ .



of sternum and left femur. *Microscopically*, intense leukæmic infiltration of splenic pulp, liver sinusoids and bone marrow; less intense infiltration in kidneys. No myeloid metaplasia discovered in the viscera. A differential marrow-cell count showed great preponderance of myeloblasts.

TABLE III  
Case V. *Hæmatological findings*

Date 1946/47	Hb. (per cent.)	R.B.C. 10 <sup>6</sup> /c.mm.	W.B.C. per c.mm.	Poly. (per cent.)	Lymph. (per cent.)	Mono. (per cent.)	Nucleated red cells		
							Total per 100 W.B.C.	Percentage megaloblasts	
30/12	44	1.4	1750	23	65	12	160	70	Reticulocytes 4.3 per cent.
10/1	44	1.8	...	...	...	...	...	...	Reticulocytes 12.0 per cent. on 7th day of liver
13/1	38	...	2200	28	65	7	150	...	...
16/1	48	1.9	2100	30	62	8	28	70	After transfusion
30/1	38	1.75	4600	24	69	7	85	53	...
3/2	25	1.1	4200	32	65	3	30	80	Platelets 33,000 per c.mm.
10/2	62	...	...	...	...	...	...	...	Reticulocytes 0.6 per cent. Trans- fused 4 pints
14/2	...	...	9000	36	64	—	15	—	Reticulocytes 2.0 per cent. on 6th day of folic acid
17/2	...	...	9800	31	66	3	5	—	...
19/2	52	2.8	23,800	...	...	...	...	...	...
20/2	...	...	28,500	18	45	15?	2	—	Also 3 per cent. mye- loblasts, 13 per cent. myelocytes, 6 per cent. metamyelo- cytes
22/2	...	...	37,200	...	...	...	...	...	See text
Sternal marrow differential percentages									
							3/1/47 (biopsy)	23/2/47 (1 hr. after death)	
Reticulum cells . . . . .							...	1.0	
Blasts . . . . .							4.0	57.0	
Promyelocytes . . . . .							...	1.0	
Myelocytes . . . . .							5.5	5.0	
Metamyelocytes . . . . .							2.5	7.0	
Neutrophil polymorphs . . . . .							3.0	6.0	
Lymphocytes . . . . .							1.0	7.0	
Pro-erythroblasts . . . . .							5.0	2.0	
Normoblasts (A . . . . .							0.0	4.0	14.0
(B . . . . .							3.0	5.0	
(C . . . . .							5.0	5.0	
Megaloblasts (A . . . . .							17.0	71.0	Nil
(B . . . . .							41.0		
(C . . . . .							13.0		

Cases IV and V are related and may be considered together. The former is that of a woman with an illness of 12 years' course which must be regarded as pernicious anæmia in spite of the inadequacy of the earlier hæmatological findings. Presenting symptoms included



anæmia, glossitis and paræsthesia. She had gastric achlorhydria. Liver injections maintained her in good health apart from one relapse when treatment lagged. The terminal acute leukæmia lasted at most 5 months. The association of these two diseases may, of course, be a matter of chance, as Sterne *et al.* (1941) suggest. Abnormal granulocyte formation was, however, observed in the marrow of this patient before the blood became leukæmic and was thought to be a relic of the marrow changes of pernicious anæmia. Giant metamyelocytes and bizarre nuclear forms, imperfect cytoplasmic granulation and vacuolation of the early myeloid cells were seen. Dameshek and Valentine (1937) state that in pernicious anæmia the abnormal granulocytes disappear from the marrow under the influence of liver therapy but less rapidly than the megaloblasts. Foy and Kondi (1943) think that the giant metamyelocyte is as characteristic of pernicious anæmia as the megaloblast. Jones (1937) believes that abnormalities are to be found in all stages of granulocyte development in this disease, a view with which we agree. Moreover, we feel some doubt whether the common giant leucocyte of pernicious anæmia is indeed a metamyelocyte. There is a possibility that these cells are direct but abnormal descendents of the myeloblast, *i.e.* that they may lie outside the normal myeloblast—myelocyte—metamyelocyte sequence, in analogy with the megaloblastic deviation of the red cell precursors. We have seen morphologically indistinguishable cells in the marrow of a case of acute aleukæmic myelogenous leukæmia without macrocytic anæmia and in these surroundings they appear to be more nearly related to the myeloblast than to the normal metamyelocyte. They differ from the monocytoid myeloblast described by Naegeli in acute leukæmia only in their more prominent granulation.

The acute leukæmia in both case IV and case V was marked by the circulation of monocytoid myeloblasts, but evidence is lacking to relate this type of acute leukæmia specifically with the pattern of abnormal granulocyte development seen in pernicious anæmia. Although the view has been expressed by Davidson and Gulland (1930) and by Jones (1937) that deficiency of liver principle may account for imperfect maturation of granulocytes as well as erythrocytes, we are not in a position to say that such a deficiency played a direct part in the pathogenesis of the leukæmia in these two cases. We are here more concerned in recording the fact that in these two instances of acute leukæmia there was an associated disorder of the red-cell-forming tissues of pernicious anæmia type. Such an association accounts for the syndrome of leukanæmia, a subject already adequately reviewed by Drysdale (1907-08), Sterne *et al.* (1941) and Foy, Kondi and Murray (1946). The last-named authors and also Treadgold (1913) each describe a case very similar to our case V.

## DISCUSSION

The cases here recorded have been chosen from our recent experience to illustrate the more than coincidental association of leukæmia and anæmia. It seems to us that sufficient attention has not been paid to the matter or to the implications arising from cases of leukanæmia and other anomalous types which occur from time to time. Most authors (*e.g.* Forkner, 1938, p. 54; Whitby and Britton, 1946; Wintrobe, 1946, p. 707) assume that anæmia in leukæmia ordinarily arises from crowding out or replacement of erythropoietic by leucopoietic tissue in the bone marrow. This view implies an analogy with other space-occupying lesions of bone marrow which produce leuco-erythroblastic anæmia. But Vaughan (1936) does not regard destruction or displacement of red-cell-forming tissue as the essential cause of this type of anæmia in such conditions as osseous carcinoma, myelomatosis and osteosclerosis, in the former of which, at least, there is a strong probability that the total amount of red marrow in the body is actually increased. She ascribes it to a stimulated though possibly disordered erythropoiesis competing with an accelerated loss or destruction of red cells, the nature of which is unknown. Vaughan and Harrison (1939), however, report two cases of myelosclerosis with leuco-erythroblastic anæmia in which the red cells were abnormally fragile to hypotonic saline. In leukæmia, also, it has been suspected that excessive destruction of red cells may explain the anæmia and that the erythroblastosis may be similar in nature to that found in other hæmolytic anæmias. Jaffé (1935) noted that erythropoiesis was often nearly as prominent a feature of the marrow in myelogenous leukæmia as leucopoiesis. Especially was this the case in acute myelogenous leukæmia, and Jaffé went so far as to suggest that initial destruction of red cells might be of importance in the pathogenesis of this disease. Hæmosiderosis of the viscera in leukæmia has been noted by Jaffé and by von Kress (1933-34), but Whipple and Robschey-Robbins (1933) failed to find any significant increase in the iron content of the liver in acute leukæmia. Siderosis has not been conspicuous in any of our own autopsy material. Watson (1938) found conflicting evidence in cases where pigment excretion was studied and concluded that a hæmolytic anæmia might occur in association with leukæmia, but that this was by no means the rule. Actual blood loss may contribute to the severity of the anæmia. The hæmorrhagic tendency, however, usually becomes marked only in the later course of the disease where there is thrombocytopenia or perivascular leukæmic infiltration (Forkner, p. 55).

We are bound to agree with the broad summing-up of Forkner (p. 55) that disorderly blood formation, blood loss and increased blood destruction may each play a part in the mechanism of anæmia in leukæmia, but our own studies lead us to think that the two last-named are only occasional and contributory factors and that disturbance of

normal erythropoiesis is not only a common accompaniment but an essential pathological feature of the disease. Moreover, in our view, erythrogenic tissue is affected in different ways depending upon the type of leukaemia.

In myelogenous leukaemia nucleated red cells are released from the marrow almost constantly, irrespective of the degree of anaemia. Instances are also known of the co-existence of chronic myelogenous leukaemia and erythraemia in the same patient or of the transmutation of one disease into the other. Forkner, who has reviewed this subject, refers to such cases under the term erythroleukaemia. We have encountered a few examples of this combination but we have no new data worth detailing. So far as we know erythraemia with erythroblastosis has not been found in association with lymphatic leukaemia with anything like the same frequency. The best documented cases of leukaemia have all been examples of erythrocyte disturbances of pernicious anaemia pattern occurring in association with myelogenous leukaemia, usually of the acute type. Richter (1938) also notes that these two abnormalities of the red cells both occur predominantly in myeloid leukaemia. Stimulated activity of the erythron in myelogenous leukaemia would appear probable, having regard to the close histo-anatomical relationship of the red- and granular-cell-forming tissues. It is virtually impossible to judge by histological methods whether the total amount of erythropoietic marrow in the body is increased, but we suspect this to be the rule in myelogenous leukaemia. Assuming then that red-cell-forming tissue is hyperplastic and its activity stimulated in this disease, anaemia when it supervenes must be due either to blood loss or accelerated blood destruction or to a lessened rate of delivery of mature erythrocytes. The first we have already described as only an incidental cause. Direct evidence of increased haemolysis is generally wanting. The probability therefore remains that, in spite of hyperplasia of the erythrogenic tissue, fewer red cells are actually produced. This cannot easily be demonstrated, but an analogy may be drawn from pernicious anaemia and from hypochromic microcytic anaemia (Wintrobe, p. 539), in both of which erythroid hyperplasia of the marrow is prominent and the anaemia is known to be due to interference with erythrocyte maturation.

In acute leukaemia anaemia is constant and increases rapidly in severity. The cases we have studied have been of acute myeloblastic type. In the two examples here recorded (cases IV and V), anaemia and active marrow erythropoiesis were shown to be present immediately before leukaemia was demonstrable in either blood or marrow. In one case there was a long history of classical pernicious anaemia and in the other recent severe anaemia with megaloblastosis of both bone marrow and blood. In discussing these cases we have pointed out that the pernicious anaemia principle failed to influence the course of the leukaemia although it brought about normoblastic reversion of the marrow. It is our view that the striking anaemia of acute leukaemia

results from abnormal functioning rather than ablation of the red-cell-forming marrow, though the abnormality may not always take the form of a megaloblastic reaction. It is probable that cases such as we have described and other instances of leukanæmia represent more than a coincidence of two diseases and that they are the outcome of some unknown stimulus affecting both types of marrow tissue simultaneously or one before the other. These cases may be a connecting link between the purer forms of acute leukæmia and those much rarer examples of immature cell erythræmia such as that described by Israëls (1939a). It is certain that in both acute and chronic myelogenous leukæmia erythropoiesis and leucopoiesis should not be considered apart from each other and it is inaccurate to dismiss the anæmia in these diseases simply as a secondary phenomenon.

The anæmia in chronic lymphatic leukæmia, on the other hand, though it tends to be more pronounced at the time of diagnosis and is often very severe towards the end, is accompanied much less constantly by signs of stimulated erythroid activity. There is a lesser frequency of erythroblastosis. Crowding of the bone marrow by lymphocytes reduces both its red- and its granular-cell-forming potentialities. The anæmia in lymphatic leukæmia seems to be due, in most cases, to depression of erythropoiesis without compensatory stimulation.

### SUMMARY

1. Both the incidence and the severity of leukæmic anæmia are found to be highest in acute leukæmia and higher in chronic lymphatic than in chronic myelogenous at the time of first diagnosis.

2. Nucleated red cells are less frequently present in the blood in chronic lymphatic leukæmia than in the other types of the disease and severe grades of anæmia in this condition appear to be due mainly to lymphocytic infiltration of the bone marrow with hypoplasia of the erythron.

3. In both chronic and acute myelogenous leukæmia there is evidence of stimulated and abnormal activity of red-cell-forming tissue. In some cases this may antedate other manifestations of leukæmia by a long interval of time. Peculiar signs of disordered erythropoiesis are seen in cases of erythroleukæmia on the one hand and leukanæmia on the other.

4. Blood loss and excessive red-cell destruction may operate in some cases to aggravate the anæmia but its essential nature appears to be, in lymphatic leukæmia, hypoplasia of the erythron, and in myelogenous leukæmia defective production of cells from a hyperplastic marrow.

5. The incidence and severity of anæmia in 50 cases of leukæmia at the time of first diagnosis have been analysed and five illustrative cases of particular interest are recorded.

We gratefully acknowledge our debt to Dr J. R. H. Towers and Prof. R. E. Tunbridge for access to their patients and their clinical notes and to Miss E. Thorley and Mr W. H. B. Vincent for their help with the pathological technique and records.

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# THE MECHANISM OF PARENCHYMATOUS DEGENERATION PRODUCED BY DIPHTHERIA TOXIN

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(PLATES XIX-XXI)

It is nearly 100 years since Virchow (1852) described "parenchymatous inflammation"—classified later as degeneration—and yet the exact mechanism of this condition is still unknown. Virchow (1858) envisaged parenchymatous degeneration as being due to a state of stimulation of the cells, in which the tissues have taken up excess of nutrient materials. Davidman and Dolley (1920-21) also thought that cloudy swelling was due to a process of stimulation. The literature of the subject subsequent to Virchow's publications is very extensive and need not be reviewed here. It has been suggested that the appearance of the cells in parenchymatous degeneration might be due to an increased hydration of protoplasmic proteins (see discussion following the article by Bell, 1913; Uher, 1931, 1932, 1933), although definite evidence on the question is lacking. It was hoped that by studying water and electrolyte distribution in the tissues this hypothesis might be tested and it is believed the data here presented show in a quantitative manner that in parenchymatous degeneration, produced by diphtheria toxin, there is increased hydration of protoplasm.

## MATERIAL AND METHODS

Human post-mortem material is unsuitable for the type of investigation planned because of unavoidable autolytic tissue changes and rapid diffusion of electrolytes across cell membranes after death. For this reason experiments were carried out on rabbits injected with diphtheria toxin.

Albino rabbits of 1700-2300 g. weight and bred in the department were used. For each experimental animal a litter-mate was used as control. In one set of experiments the rabbits were injected subcutaneously, between the scapulae, with a dose of diphtheria toxin which would kill a 2.5 kg. animal in 4 days, i.e. approximately 1 M.L.D. for our rabbits. When these large doses of toxin were employed, the animals were killed 24 hours after the injection, at a time when they were still vigorous. They were given Nembutal (1 ml. of a 10 per cent. solution/5 lb. body weight) intravenously and were bled from the aorta. The liver and kidneys were quickly excised, weighed and samples

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taken for chemical analysis and microscopical examination. In the second set of experiments the rabbits received subcutaneously 25 per cent. of the M.L.D. and were killed 2, 3, 9 and 14 days after injection. Here also litter-mates were used; they were all injected at the same time and one was killed 2 or 3, the other 9 or 14 days later.

*Histological methods.* Sections were stained with haematoxylin and eosin, Best's ammoniacal carmine for glycogen, and by Altmann's method for mitochondria; frozen sections were stained with Scharlach R for fat.

*Chemical analyses.* The water, Cl, Na and K content of tissues and serum and the non-protein- and total-nitrogen content of tissues were determined. All values were calculated on the basis of fat-free fresh tissue; this eliminates variations due to different fat content of the tissues from animal to animal.

For the determination of water content weighed samples of tissue and serum were frozen with acetone- $\text{CO}_2$  snow mixture and dried *in vacuo* over  $\text{P}_2\text{O}_5$ ; the residual moisture was removed by heating in an oven at  $103^\circ\text{C}$ .

For the extraction of fat (mostly neutral fat) the dried tissues were covered with ether; after 3-4 hours the ether was drawn off and fresh solvent added, which was removed 3 hours later and replaced by light petroleum (b.p.  $40^\circ$ - $60^\circ\text{C}$ .). After standing overnight, the extraction with light petroleum was repeated 5 times during the next 24 hours, after which the organic solvent remaining in the tissues was evaporated off by heating in the oven at  $103^\circ\text{C}$ . The weight after extraction was taken as the weight of the fat-free solids in the sample. For the electrolyte determinations the fat-free dried tissues were powdered to ensure uniform sampling.

Chlorine was determined by the method of Wilson and Ball (1928).

For the determination of sodium and potassium, 1.5-2.0 g. of tissue powder were weighed into a silica beaker, 5 ml. of 4N.  $\text{H}_2\text{SO}_4$  added and the whole heated at  $110^\circ\text{C}$ . in an oven overnight. The residue was then ashed in a muffle furnace at  $500^\circ\text{C}$ . The ash was dissolved in 5 ml. of N.HCl and transferred quantitatively to a 50 ml. volumetric flask and made up to volume. One ml. of this was placed in a centrifuge tube, evaporated to dryness in an oven and used for the determination of potassium as the chloroplatinate by the method of Salit (1940). 0.5 g. of solid CaOH was added to the remainder in the volumetric flask to remove phosphates; after 30 min. the mixture was centrifuged and 20 ml. samples of the supernatant were measured into beakers containing 2 ml. of N.HCl. The contents of the beakers were concentrated on a water-bath to approximately 2 ml. and the precipitation of sodium with uranyl-zinc-acetate was carried out according to the method of Butler and Tuthill (1931). The electrolytes in the serum were determined by the same methods.

Nitrogen was determined by a micro-Kjeldahl method. When the digestion mixtures became clear after heating with 4 ml. of a 1 per cent. (w/v) selenium dioxide solution in 50 per cent. (v/v)  $\text{H}_2\text{SO}_4$ , the flasks were cooled, a few drops of ammonia-free water and  $\text{H}_2\text{O}_2$  (100 vols., B.D.H. micro-analytical reagent) added and the heating continued for a further 2 hours. For the determination of non-protein N approximately 2 g. of fresh tissue were ground with 10 per cent. trichloroacetic acid solution (25 ml./g. tissue) and samples of the filtrate taken for digestion. Total N was determined on 20-30 mg. samples of fat-free dry tissue powder.

## RESULTS

### *Morphological observations*

Macroscopically there were but few changes in the experimental animals. There were petechial hæmorrhages and necroses in the subcutaneous tissues and dorsal muscles, but only at the site of

## PARENCHYMATOUS DEGENERATION

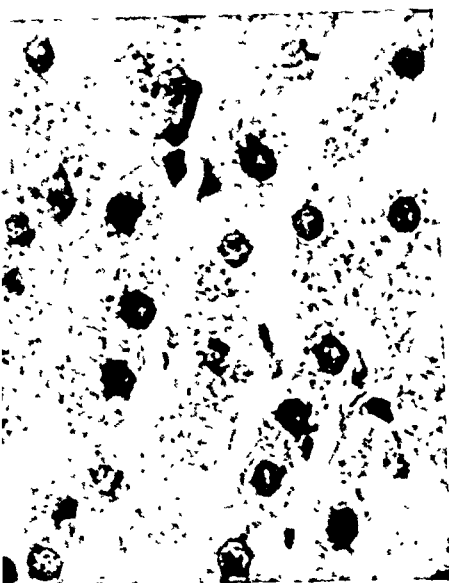


FIG. 1.—Normal rabbit liver, showing dark-staining nuclei and coarsely granular cytoplasm. Hæmatoxylin and eosin.  $\times 660$ .

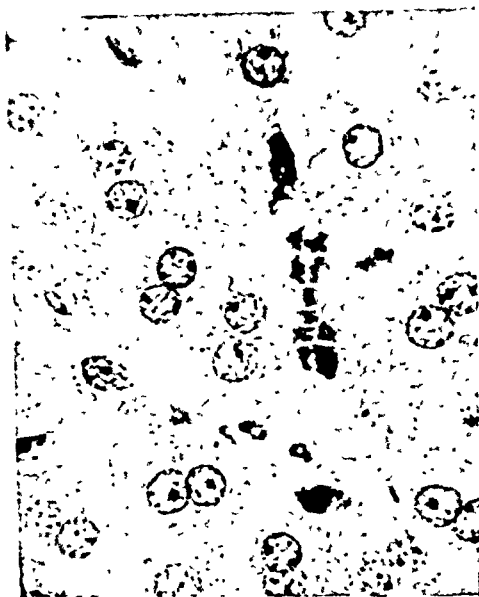


FIG. 2.—Rabbit liver 24 hrs. after the injection of 1 M.L.D. of diphtheria toxin. The cells are swollen and the nuclei stain much paler and are larger than in fig. 1. The cytoplasm shows fine granularity. Hæmatoxylin and eosin.  $\times 660$ .



FIG. 3.—Normal rabbit liver stained for mitochondria, which appear as discrete granules in the cytoplasm.  $\times 720$ .

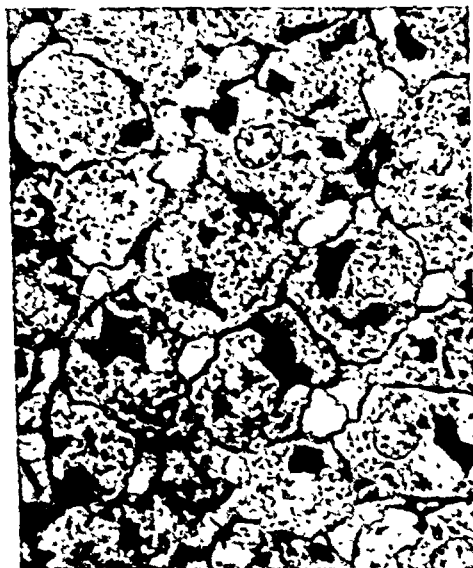


FIG. 4.—Rabbit liver stained for mitochondria 24 hrs. after the injection of 1 M.L.D. of diphtheria toxin. The mitochondria show conglomeration into large granular masses. The swelling of the cells is very obvious as compared with fig. 3.  $\times 720$ .





injection. In the experiments with the larger dose of toxin there were occasionally a few punctate hæmorrhages in the omentum and hyperæmia or small hæmorrhages in the adrenals 24 hours after the injection. With the smaller dose of toxin only the adrenals showed these changes. There were no definite changes to the naked eye in the liver. The renal cortex was usually swollen and the glomeruli stood out as tiny red dots. The blood content of liver and kidneys was determined in several experiments by the method of Childs and Eichelberger (1942) but no increase was found as compared with the controls.

Perhaps the most tangible gross change in the liver and kidneys was their increased weight. Table I shows the mean weights (g./100 g.

TABLE I

*Liver and kidney weights (g./100 g. body weight) of control and diphtheria toxin-treated rabbits*

Organ	Controls	Experimental	Standard error of difference of means
Liver . . . .	$2.83 \pm 0.28$ (10)*	$3.34 \pm 0.27$ (13)	$\pm 0.131$
Kidney . . . .	$0.480 \pm 0.091$ (10)	$0.580 \pm 0.097$ (16)	$\pm 0.038$

\* Figures in brackets indicate the number of animals used.

body weight) of the liver and kidneys of the control and experimental animals; the difference of the means for the liver is definitely, and for the kidneys probably, significant.

Histological examination of the tissues was carried out on each animal in order to ascertain the type of lesions produced and to correlate these with the chemical findings. Fig. 1 illustrates the usual histological appearance of the liver of normal rabbits; the nuclei stain intensely and the cytoplasm shows a rather coarse granularity owing to the presence of glycogen, which is unstained. Fig. 2 shows the typical appearances either 24 hours after the lethal dose of toxin or 72 hours after the non-lethal. The nuclei and cytoplasm stain very faintly and both appear swollen as compared with the normal. There is either no glycogen or only a trace in the cytoplasm. Doubly nucleated cells are a prominent feature in the experimental animals. By cell counts it is found that in the liver of normal rabbits about 10 per cent. of the cells have two nuclei; in the experimental animals 30 per cent. or even more, of the liver cells are double-nucleated but karyokinesis is not observed. In normal livers the mitochondria are distributed in the cytoplasm as discrete granules (fig. 3); in the toxin-treated animals they show a tendency to crowd around the nuclei and often to conglomerate into larger masses (fig. 4).

The renal cortical tubules of the rabbits injected with toxin are swollen, the granular cytoplasm and nuclei stain much paler than normal (figs. 5 and 6), and the normally rod-shaped mitochondria of the proximal convoluted tubules become fragmented (figs. 7 and 8). These changes in mitochondria were described in 1914 by Dibbelt. Neither in the kidneys nor in the liver is stainable fat observed in excess of that found in the controls.

These histological changes would probably be accepted by most pathologists as parenchymatous degeneration, but whether they are exactly identical with the changes described originally as cloudy swelling is more difficult to decide. In fresh preparations of suspensions of single liver cells in an isotonic  $\text{PO}_4^-$  buffer, pH 7.4, viewed through the microscope, the only difference between normal liver cells and cells isolated from the liver of experimental animals is the larger size of the latter and the frequently observed double nuclei. Both normal and degenerate cells contain occasional refractile globules (fat droplets) and opaque granules in about equal numbers; it is only a subjective impression that these opaque granules are somewhat larger in the degenerate liver cells, an observation which might be related to the conglomeration of mitochondria (*cf.* fig. 4).

#### *Results of chemical investigations on the liver*

From recent investigations it is known that chlorine and sodium in muscle are chiefly extracellular and are in membrane equilibrium with the ions in plasma; potassium on the other hand is intracellular (*cf.* Manery and Hastings, 1939; Höber, 1945). From the determination of the concentration of chlorine in plasma or serum water and from the water and chlorine content of the tissues, then, it is possible to calculate the volume of the extracellular, or more correctly the Cl-space, and also to arrive at the concentration of water in the intracellular phase. It seems probable that this is also true of the liver (Manery and Hastings) but certainly not of the kidney, where excretion of these electrolytes takes place. The water and electrolyte determinations were carried out with these considerations in mind.

The size of the extracellular phase ( $V$ , g./kg. fat-free fresh tissue) and the water content of the intracellular phase ( $C$ , g./kg. intracellular phase) of the liver were calculated by formulæ (1) and (2) (Hastings and Eichelberger, 1937):

$$V = \frac{\text{mEq. Cl}^-/\text{kg. fat-free fresh tissue}}{\frac{0.99}{0.95} \text{mEq. Cl}^-/\text{kg. serum water}} \quad . \quad . \quad . \quad (1)$$

$$C = \frac{\text{H}_2\text{O g./kg. fat-free fresh tissue} - 0.99 \times V}{1000 - V} \times 10^3 \quad (2)$$

The data required for these calculations are summarised in table II. The analytical results and the calculations based thereon for the

PARENCHYMATOUS DEGENERATION

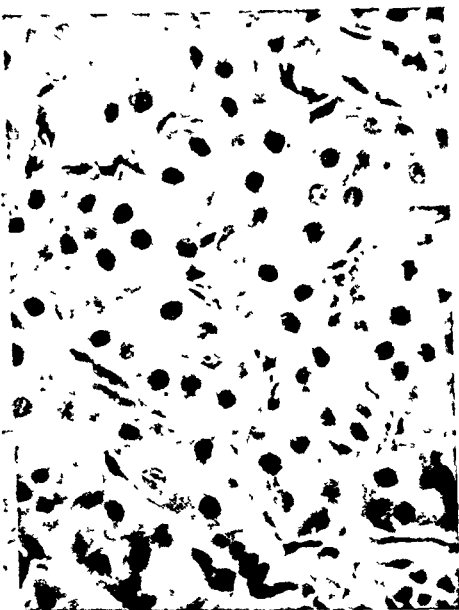


FIG. 5.—Normal proximal convoluted tubules of rabbit's kidney. Hæmatoxylin and eosin.  $\times 410$

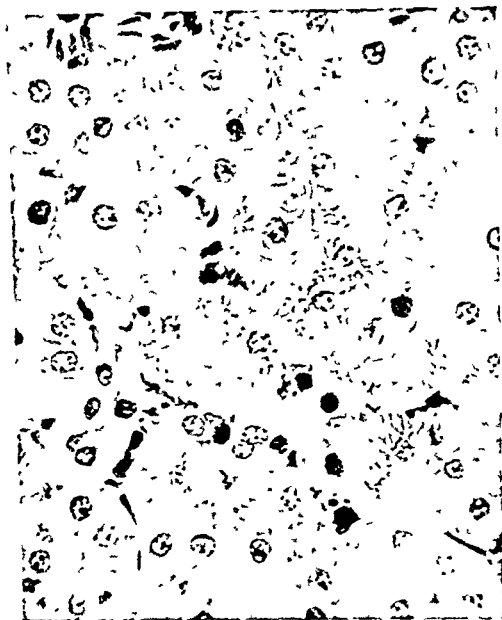


FIG. 6.—Rabbit kidney 24 hrs. after the injection of 1 M.L.D. of diphtheria toxin, showing proximal convoluted tubules. The granular cytoplasm and nuclei stain paler than normal. Hæmatoxylin and eosin.  $\times 410$ .

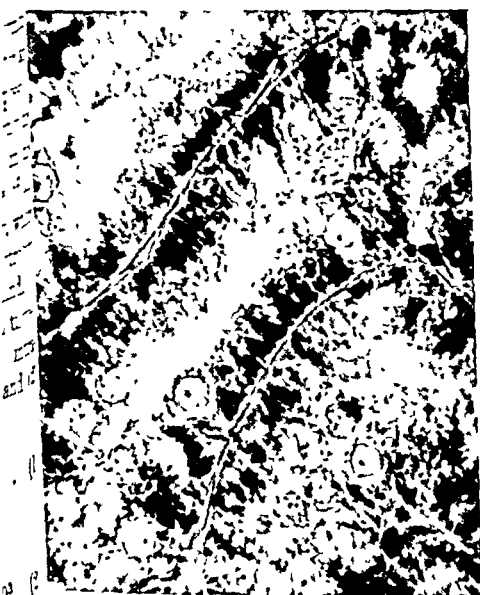


FIG. 7.—Normal rabbit kidney, illustrating the rod shaped mitochondria of the proximal convoluted tubules.  $\times 900$ .



FIG. 8.—Rabbit kidney 24 hrs after the injection of diphtheria toxin, showing fragmented mitochondria in the proximal convoluted tubules.  $\times 900$ .



control animals are in very good agreement with the data of Manery and Hastings. It appears that in parenchymatous degeneration the

TABLE II

*Chlorine content of serum and water and electrolyte content of liver of normal and diphtheria toxin-treated rabbits; extracellular phase and water content of intracellular phase of liver. All tissue constituents refer to those of fat-free fresh liver*

	Serum Cl (mEq./kg.)	Water content of liver (g./kg.)	Liver electrolytes			Extra- cellular phase (g./kg.)	Water in intracellular phase (g./kg. intracellular phase)	Remarks
			Cl	Na	K			
			(mEq./kg.)					
Mean of 10 controls	111.7	719	29.0	31.0	82.0	248	630	Normal
S.D.	±3.1	±8	±2.4	±2.6	±4.5	±20	±18	
Mean of 10 experiments	111.2	770	26.3	28.6	75.6	225	702	24 hours after injection of
S.D.	±3.7	±15	±3.4	±3.9	±2.5	±28	±18	1 M.L.D. of diphtheria toxin
Standard error of difference of means	±1.6	±5.7	±1.4	±1.6	±1.7	±12	±8.5	

water content of the organ has increased, but this increase accounts only partly for its increase in weight. As will be shown later, most of the enlargement of the liver is due to an increase in the protein content. The chloride space, or extracellular phase, is slightly smaller in the experimental animals than in the controls, but the difference is not statistically significant. The calculation of the water content of the intracellular phase shows, on the other hand, that the increased water content of the whole organ is brought about by an increase in the amount of intracellular water. On the basis of histological evidence it is inferred that the excess water is uniformly distributed throughout the cytoplasm, *i.e.* there is an "intracellular oedema".

The pertinent results of the experiments with the non-lethal doses of toxin are shown in table III. Seventy-two hours after the injection of 0.25 M.L.D. of toxin the histological changes in the liver and kidney are indistinguishable from those seen 24 hours after the injection of 1 M.L.D. of toxin. The chemical analyses also yielded similar results, *i.e.* an increased water content due to "intracellular oedema". When the animals were allowed to survive 9 or 14 days, a gradual recovery took place, the water content of the whole organ and of the intracellular phase returning to normal by the 14th day. Histologically also the livers are normal by the 14th day, except that the liver-cell columns appear separated by a wider space than usual. This can be well correlated with the increased extracellular space calculated from electrolyte distribution (see table III).

Forty-eight hours after the injection of the non-lethal dose of toxin the results of both histological and chemical investigations were quite different from those seen in parenchymatous degeneration. The water content of the liver was increased to approximately the same extent as in cloudy swelling and there was an increase in the amount of intracellular water, but there was a great reduction of the extracellular space also (see table III). In hæmatoxylin and eosin sections

TABLE III

*The effect of non-lethal doses (approximately 0.25 M.L.D.) of diphtheria toxin on the water content and intra- and extracellular phases of the rabbit's liver. All constituents refer to those of fat-free fresh liver*

Expt. no.	Water content (g./kg.)	Extra-cellular phase (g./kg.)	Water content of intracellular phase (g./kg. intracellular phase)	Time after injection of toxin	Microscopical changes
Mean of 10 controls	719	248	630	...	Normal
S.D.	±8	±20	±18		
11	742	187	715	48 hours	Glycogen storage
12	764	197	709	" "	
13	757	175	708	" "	
14	760	176	709	" "	
Mean	756	184	710	48 hours	...
15	763	242	690	72 hours	Parenchymatous degeneration
16	753	266	668	" "	
17	764	223	699	" "	
18	771	227	706	" "	
Mean	763	239	691	72 hours	...
19	741	193	682	9 days	Gradual recovery
20	741	196	680	9 "	
21	723	295	611	14 "	
22	734	298	625	14 "	

the liver cells, with well-defined cell membranes, appear blown up and tightly packed together (fig. 9), which agrees with the finding of a decreased extracellular space. The empty appearance of the cytoplasm in these sections is due to distension of the cells by large numbers of coarse granules of glycogen (fig. 10). These changes are probably due to the effect of toxin on the adrenals and are manifestations of an "alarm reaction". They will be discussed later.

Having established the fact that in parenchymatous degeneration there is an intracellular œdema, the question remained as to how the increase in the intracellular water was brought about. There are two obvious possibilities: (a) that the toxin causes a breakdown of cytoplasmic protein and the resulting smaller molecules increase the intracellular osmosis; and (b) that, due to alteration in the cell membrane, its permeability to water is increased. Both these

possibilities were explored but very little positive information has been gained.

It was thought that if there is increased protein breakdown in the liver, there might be an increase in the non-protein-N content of the organ. The non-protein- and protein-N content of the liver of control rabbits and of those injected with 1 M.L.D. of toxin are shown in table IV.

TABLE IV

*Non-protein- and protein-N content of liver of control rabbits and those injected with 1 M.L.D. of diphtheria toxin*

	Non-protein-N (mg./100 g. fat-free fresh liver)	Protein nitrogen		Time after injection
		mg./100 g. fat-free fresh liver	mg./100 g. rabbit	
Mean of 10 controls, with S.D.	218 ± 15	2931 ± 234	81.8 ± 4.25	24 hours
Mean of 10 experiments, with S.D.	236 ± 40	2887 ± 105	98.3 ± 6.71	
Standard error of difference of means	± 14.3	± 86	± 2.65	

It appears that the non-protein-N content of the experimental livers did not differ significantly from that of the controls, although the greater standard deviation indicates that the diphtheria toxin had some slight but variable effect. The concentration of protein was unaltered, but taking into account the enlargement of the livers (cf. table I) the absolute amount of liver protein was increased by about 20 per cent. This finding is in excellent agreement with the histological evidence of increased cellular division in the toxin-treated animals and also accounts for the enlargement of the liver. The proteins newly deposited in the liver might have a lower molecular weight than those found in the normal organ, and in that case increased intracellular osmosis could explain the intracellular oedema. No evidence, however, could be obtained on this point and the question must be left open.

In an attempt to investigate the properties of the liver cell membrane, the electrophoretic mobility of single liver cells suspended in an  $M/15 \text{ PO}_4^{\equiv}$  buffer, pH 7.4, was determined in the microscopical electrophoresis cell of Abramson (Abramson, Moyer and Gorin, 1942). The electrophoretic mobility of such large particles as the liver cell depends entirely on the quality, or more precisely on the net electric charge, of the surface membrane.

The suspensions of liver cells were made with the aid of a special syringe devised and kindly lent to me by Dr Roland John, who uses it to prepare single-cell suspensions of rat tumours for intravascular injection. He informs me that the cells remain viable. A detailed description of this syringe and of



its use will be given by him. It consists essentially of a duralumin barrel fitted with a piston moving along a screw thread. Instead of a nozzle the syringe is provided with a stainless steel plate having a small hole in its centre; the diameter of this hole on the inside is  $150\ \mu$  and on the outside  $50\ \mu$ . Thus there is a funnel-shaped bore through the plate. Scrapings made with a scalpel from a cut surface of the liver are placed in this syringe and squirted through the hole of the front plate. The tissue pulp collected on a watch-glass is transferred with a Pasteur pipette into a tube containing the buffer and distributed in it by blowing air through. The tube is left standing for about 1 minute, during which the coarser clumps of liver cells separate out. The supernatant is transferred to a centrifuge tube and placed in the centrifuge which is rapidly accelerated to about 2000 R.P.M. and stopped. The individual liver cells sediment down into a small pad; the supernatant, containing cell debris and nuclei, is discarded. The sedimented cells are re-suspended by agitation in fresh buffer. A portion of this specimen is further diluted and used for filling the electrophoresis cell. Most of the individual liver cells in such a suspension appear intact, with well-defined cell membrane, cytoplasmic granules and nucleus; they are usually spherical or oval. Only damaged cells, easily distinguishable, have irregular outlines. Cells from normal livers have an electrophoretic mobility (at room temperature) of  $1.50 \pm 0.17\ \mu/\text{sec.}/\text{volt/cm.}$ , those from livers of toxin-treated animals  $1.47 \pm 0.17\ \mu/\text{sec.}/\text{volt/cm.}$  By comparison, the author's red corpuscles under identical conditions had an electrophoretic mobility of  $1.50\ \mu/\text{sec.}/\text{volt/cm.}$  All liver cells from the same animal had identical mobilities. There was only one animal in which, 14 days after the injection of 0.25 M.L.D. of toxin, liver cells with two mobilities were found, one with a mobility of  $1.35\ \mu/\text{sec.}$  and another type with a mobility of  $1.00\ \mu/\text{sec.}$  Histologically this liver appeared normal (recovered) and the identity of the two types of cell with different mobilities could not be established. These findings indicate only that there is no gross alteration in the physico-chemical composition of the liver cell membrane as a result of treatment with diphtheria toxin, but still do not exclude the possibility of an increased permeability of the plasma membrane to water.

### *Results obtained on kidneys*

The number of analyses on kidneys was comparatively small and therefore figures of individual experiments are shown in table V instead of the summary presentation used in the case of the liver. The kidney, because of its complex structure, cannot be considered as consisting merely of extra- and intracellular phases, but more likely of extracellular, "intratubular" and intracellular phases. Nor is it justifiable to calculate the volume of such phases from simple electrolyte distributions between plasma and tissue because of the excretion of salts. Any changes in the excretory function of the kidneys brought about by the diphtheria toxin might cause great changes in the electrolyte concentration of the kidney irrespective of changes in extra- or intracellular phases. That there was some disturbance of kidney function was evident from the slight proteinuria which was found in all animals injected with toxin.

The results, however, indicate a slight increase in the water content of the kidney and a marked fall in chlorine and sodium content. Whether the latter indicates a reduction in extracellular and "intra-

PARENCHYMATOUS DEGENERATION

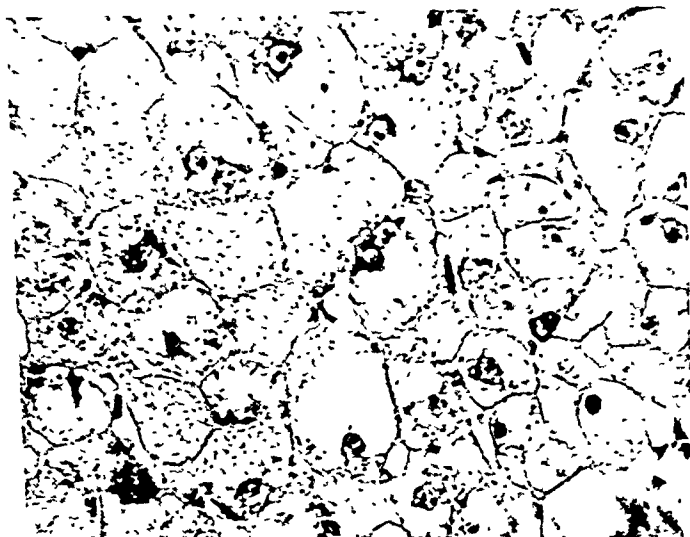


FIG. 9.—Liver of rabbit 48 hrs. after the injection of 0.25 M.L.D. of diphtheria toxin. Hæmatoxylin and eosin.  $\times 460$ .

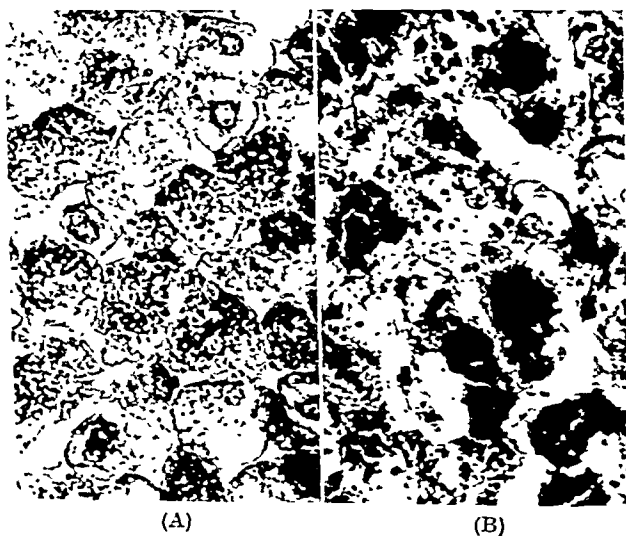


FIG. 10.—(A). Glycogen in normal rabbit liver. (B). Glycogen in the same section as illustrated in fig. 9.  $\times 460$ .



tubular " phases is uncertain, but it might be of interest to mention without going into detail that the Na and Cl excretion of rabbits

TABLE V

*Water and electrolyte content of kidneys of rabbits injected with 1 M.L.D. of diphtheria toxin as compared with controls*  
*Constituents refer to those of fat-free fresh tissue*

Animal no.	Water content (g./kg.)	Cl      Na      K (mEq./kg.)			Time after injection
Control 1	795	70.4	74.2	58.0	
" 2	785	63.0	78.3	65.0	
" 3	800	66.0	...	...	
" 4	789	62.0	71.0	65.0	
" 5	799	67.2	71.0	64.0	
Average	794	65.7	73.6	63.0	
Expt. 1	804	56.2	67.5	63.5	} 24 hours
" 2	817	50.8	64.1	57.0	
" 3	812	52.2	69.6	58.3	
" 4	812	46.7	65.0	57.4	
" 5	801	51.9	...	62.5	
" 6	808	49.8	...	63.3	
Average	809	51.3	66.5	60.3	

injected with diphtheria toxin was extremely low and that there was definite salt retention. This, like the glycogen storage in the liver, might be the result of hyperactivity of the adrenal cortex.

It is inferred, therefore, only by the analogy between the histological changes in the kidney and liver, that in the kidney also there is an intracellular oedema in parenchymatous degeneration.

## DISCUSSION

The evidence presented shows that in parenchymatous degeneration produced experimentally in the liver there is increased hydration of protoplasm and in addition a building up of new cellular elements. Whatever the point of attack of diphtheria toxin, it definitely causes increased cellular activity as evidenced by nuclear division and an increased protein content of the liver. Virchow's hypothesis that in parenchymatous degeneration the cells are in a stimulated state is therefore fully supported; further, the suggestion of an intracellular oedema is quantitatively proved. Uher (1931, 1932, 1933) studied by physico-chemical methods parenchymatous degeneration in the human liver and found that pieces of liver tissue obtained from cases of septicæmia, when soaked in isotonic NaCl or KCl solutions, gained much less weight than a piece of liver from a case of accidental death. It might be expected that, if there was already a high degree of

hydration of protoplasm in the septicæmic cases, the degree of further hydration induced by immersion in an aqueous solution would be less than with a piece of normal tissue. A piece of fatty liver would behave, however, in the same way, and it is unfortunate that Uher does not mention if these livers were histologically examined. It is uncertain, therefore, whether the quoted observation can be taken without qualification as supporting the results of the present investigation.

The observed glycogen storage in the liver 48 hours after the injection of a non-lethal dose of toxin and the salt retention clearly point to an alarm reaction. The effects of diphtheria toxin on the adrenals are well known and also that in the alarm reaction there is an increased secretion of adreno-cortical hormones (*cf.* Popják, 1944; Sayers *et al.*, 1945). Sayers *et al.* have also shown that the increased activity of the adrenal cortex in the alarm reaction is brought about by the action of the adrenocorticotrophic hormone of the anterior pituitary. Thorn, Forsham and Prunty (1947; private communication from Dr Prunty) found that the injection of adrenocorticotrophic hormone to a man suffering from mild hypopituitarism caused retention of Na and Cl and an increase in liver glycogen. While these observations may not seem to have a direct bearing on the present problem, they clearly show the complex interaction of bodily functions. Virchow's conception of degenerations as being passive processes (see Aschoff, 1921; "Virchows Lehre von den Degenerationen (passiven Vorgänge) und ihre Weiterentwicklung") seems therefore paradoxical, especially since he himself thought parenchymatous degeneration to be a response of cells to stimulation.

#### SUMMARY

1. Parenchymatous degeneration, produced in the kidney and liver of rabbits by the injection of diphtheria toxin, was studied histologically and by chemical methods.

2. By determination of the water and electrolyte content of tissues and serum, it is found that in parenchymatous degeneration of the liver there is an increased water content of the organ, and that all the excess water is intracellular. There is also increased cellular activity in the liver as evidenced by enlargement of the organ, nuclear division, and increased protein content.

3. These changes are observed 24 hours after the injection of 1 M.L.D. or 72 hours after the injection of 0.25 M.L.D. of diphtheria toxin. Two weeks after the injection of the smaller dose the condition of the liver has returned to normal.

4. Forty-eight hours after the injection of 0.25 M.L.D. of diphtheria toxin there is a great accumulation of glycogen in the liver; this, together with some other observations, is interpreted as a manifestation of an alarm reaction.

5. The kidneys of diphtheria toxin-treated animals show a slight increase in water content and a decrease in the chlorine and sodium content. It is not possible to calculate the volume of extra- and intracellular phases in the kidney from simple electrolyte distributions. It is suggested, therefore, only by analogy between the histological changes in the renal tubules and those in the liver, that cloudy swelling in the kidney is also accompanied by intracellular oedema.

I am very much indebted to Professor W. G. Barnard for his suggestions and his stimulating interest in the present work. A grant from the Thomas Smythe Hughes Medical Research Fund for the purchase of a muffle furnace is gratefully acknowledged. I wish also to thank Mrs P. de C. Williams for her skilful technical assistance and Mr A. E. Clark for the preparation of histological sections.

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## THE EFFECT OF TEMPERATURE UPON ANTI-BODY PRODUCTION IN COLD-BLOODED VERTEBRATES

K. A. BISSET

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IN bacterial infections of fish and amphibia, the balance between bacterium and host which exists at low temperatures is upset if the temperature is raised (Bisset, 1946); there is an increase in both the offensive power of the microbe and the defensive activity of the host (Bisset, 1947a). This balance is delicate and complicated, because, while immunity is much more readily acquired at higher temperatures, antibody production being inhibited below about 12° C. (Widal and Sicard, 1897; Cushing, 1942; Bisset, 1947a; and others), natural resistance is distinctly higher at low temperatures (Bisset, 1947b). Similar effects have been reported by Paillot (1921) in insects.

This complex situation may account to some extent for the anomalous reports of the effect of temperature upon bacterial diseases of cold-blooded animals. Emerson and Norris (1905) reported that the course of "red-leg" disease of frogs might be arrested by placing them at a temperature a little above freezing point. Ernst (1890), working with a similar disease of frogs, reported that their resistance to infection was greater at higher temperatures. Davis (1921-22) found that injured fish became infected at high water-temperatures.

The same anomalies have been reported among the invertebrates. Chorine (1933) reported that lowering the temperature cured infected caterpillars, whereas Pospelov (1926) produced the same effect in locusts by raising it. The available evidence is more fully discussed elsewhere in a review of the literature (Bisset, 1947c).

### *Preliminary observations*

The initial observations from which the present studies arose were made upon perch (*Perca fluviatilis*) and powan (*Coregonus clupeoides*) under natural conditions.

It is known that fish are often parasitised by what are otherwise saprophytic water bacteria (Williamson, 1929; Bisset, 1946), and it was observed that the degree of infection decreases in summer as the water temperature rises, and increases in autumn (Bisset, 1948). It was also observed that as the temperature increases the fish may rid themselves completely of certain bacterial species. If these bacteria are commonly present in the water, the fish must resist reinfection for a certain period. They are nevertheless found to be infected once more at the end of the winter. Thus the immunity which they acquire and maintain while the temperature is high must be lost when it falls.



Experiments were devised to investigate this phenomenon under laboratory conditions. The common frog (*Rana temporaria*) was employed for serological work because these animals survive handling better than fish, and the available evidence indicates that their immunological reactions are identical.

### Methods

Frogs were immunised by intramuscular inoculation in the thigh with a fine needle. Serum was taken after decapitation, a few c.c. of blood being collected drop by drop in a test tube, in four or five minutes, while the action of the heart still persisted. Clotting was slight, but the corpuscles sedimented readily and a cell-free serum was easily obtained.

It was found possible to produce agglutinins to high titre in suitably chosen specimens, but only after repeated inoculation over a period of several weeks. The final titre depended greatly upon the size and condition of the frog and end-points were difficult to determine. The agglutination reaction progressed slowly for several days at room temperature until sedimentation of the controls began to obscure the result. Accordingly a standard test was adopted, giving final dilutions of 1:5, 1:10, 1:25 and 1:50, the results being read after 24 hours at room temperature. Since frogs immunised at 20° C. normally gave agglutination at much higher titres than 1:50, a strong reaction in the final tube was recorded as 1:50+ and taken as the full positive figure. Comparison of titres in the higher ranges was not attempted, because, as will be seen below, it was irrelevant to the study of the phenomena under investigation.

### Experimental

Expt. I. One group of four frogs was kept at 8° C. and another at 20° C.; both groups were immunised on six successive days with a killed suspension of *Pseudomonas fluorescens*. The day after the

TABLE

*Effect of temperature during and after immunisation on the agglutinin titres developed by frogs in response to Ps. fluorescens*

Frog no.	Temperature (°C)		Agglutinin titre
	during immunisation	for 7 days after immunisation	
1	20	20	1:50+
2	20	20	1:50+
3	20	8	1:5
4	20	8	Nil
5	8	8	Nil
6	8	8	Nil
7	8	20	1:10
8	8	20	1:25

last inoculation the two groups were subdivided into four pairs (table). The first pair, which had been immunised at 20° C., remained

at that temperature; the second pair was transferred to 8° C. The third remained at 8°; the fourth was transferred to 20°. After a week, during which the frogs were not immunised further, they were killed and their serum was tested against the homologous organism.

Both frogs in the first pair gave agglutination at 1 : 50+, those in the third gave no agglutination. The second pair gave only 1 : 5 for one frog and nil for the other, showing that they had lost their agglutinins on transfer from warm to cold, as had been anticipated. An unexpected result was obtained with the fourth pair, which gave agglutination at 1 : 10 and 1 : 25, showing that antibodies not apparent when the animals were kept at 8° C. had been produced, without further immunisation, on their transfer to the higher temperature. It appeared possible that this result might have been due to antigen remaining in the bodies of the animals, and to examine this possibility another experiment was devised.

**Expt. II.** Six frogs were immunised at 8° C., three being given two inoculations on alternate days, the other three being given four. The day after the last inoculation in each case they were removed to 20° C. for a further week and then killed.

The serum of one frog in the first group gave agglutination at 1 : 5, the other two gave no agglutination in the dilutions tested. Those in the second group gave agglutination at 1 : 5, 1 : 5 and 1 : 10. If residual antigen had been responsible for the antibodies produced, no great difference between these groups or between them and the third pair in the previous experiment would have been expected.

The foregoing experiments were regarded as preliminary. For the succeeding experiments a standard immunisation technique was adopted. All the animals were inoculated on six alternate days and control groups were tested which provided positive and negative points for comparison. The positive control group was composed of five frogs immunised at 20° C. and tested 24 hours after the completion of the course. Their sera gave agglutination at 1 : 50+ in each case. The negative control group consisted of three frogs immunised at 8° C. This group was tested at dilutions down to 1 : 2 and gave no agglutination.

**Expt. III.** The appearance of antibodies in frogs immunised at 8° C., after their transfer to 20° C., was checked on this scale of comparison. A group of three frogs was transferred 48 hours after the completion of the course and tested after a week at the higher temperature. Their serum gave agglutination at 1 : 10, 1 : 25 and 1 : 25. In the four examples of this type of experiment, therefore, the titre of agglutinins was roughly proportional to the immunisation received before transfer, although so long as the animals remained in the cold no antibodies were detectable.

**Expt. IV.** The loss of antibodies when immunised animals were transferred from warm to cold conditions was further investigated. Two groups each of nine frogs were immunised in the manner described

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at 20° C.; after immunisation the first group was transferred to 8° C. They were then tested in batches of three after two, four and seven days. Both the first and second batches produced agglutination at 1 : 25, 1 : 25 and 1 : 10, the third at 1 : 25, 1 : 10 and 1 : 5. Thus the original figure of 1 : 50+ was greatly reduced after only two days at the lower temperature; it fell only slowly thereafter. The second group of nine was left at 20° C. and tested in batches of three after three, seven and fourteen days. All these frogs gave the full positive titre of 1 : 50+.

**Expt. V.** A group of five frogs was immunised at 20° C. and transferred for a week to 8° C., after which they were re-transferred to 20° C. for a further period of four days, killed and tested. All five gave agglutination at 1 : 50+. It may be assumed that the titre of circulating antibody in the serum of this group after a week at the lower temperature would not have exceeded 1 : 25, and was probably much less (see expt. IV), so that these figures indicate an immediate restoration of antibody titre on return to 20° C. The experiment was repeated with a further group of three frogs and identical results obtained.

**Expt. VI.** In order to discover whether the antibodies which appeared and disappeared in this manner from the serum of frogs could be detected in their tissues, the livers of three frogs immunised at 20° C. and giving serum agglutinins at 1 : 50+, and of two frogs immunised at 8° C. which gave no agglutination at 1 : 2, were ground up with sterile sand and suspended in saline. The livers (weight approximately 0.5 g. each) were suspended in 1 c.c. of saline, which, after the sedimentation of the larger particles, gave a dilution of roughly 1 : 4. The suspensions were centrifuged for a few minutes at low speed, and final dilutions of 1 : 12, 1 : 24 and 1 : 50 were made with a suspension of the homologous organism. No agglutination occurred in 48 hours at room temperature in any of these dilutions, and no difference could be detected between the reactions of the two groups.

### *Discussion*

It appears from these observations that it is possible for cold-blooded animals to acquire immunity at low temperatures, although it is not manifested until the temperature is raised. Similarly, an animal immunised at a high temperature does not exhibit immunity as long as the temperature is kept low. Two distinct mechanisms, therefore, seem to operate in the process of immunisation. The first is the acquisition of the potential for antibody manufacture, the second the actual production of antibodies and their appearance in the blood. Of the two, the production process is the more affected by temperature; unless this is sufficiently high production of antibodies is inhibited, and if these have already been produced they commence to disappear from the serum.

Thus under natural conditions immunity is presumably raised and lowered according to the seasonal changes of temperature and requires no prolonged period of immunisation to replace it when lost. This hypothesis, taken in conjunction with the greater natural resistance which such animals exhibit at lower temperatures, goes far to explain the contradictory accounts, previously mentioned, of the affect of temperature upon actual diseases of both invertebrates and cold-blooded vertebrates. If the infecting bacteria are antigenically unfamiliar to the animal the initial infection will progress more readily at high temperatures. If, on the other hand, some degree of equilibrium is already established between parasite and host, the increase in temperature will upset it, and unless the virulence of the bacteria is so greatly enhanced as to enable them to destroy the animal they will be eliminated (Bisset, 1946).

It appears possible that the techniques here initiated may be of value in the elucidation of some basic problems of immunology.

### Summary

1. Frogs immunised at 8° C. do not produce serum agglutinins. When transferred without further immunisation to 20° C. they commence to produce agglutinins.

2. Frogs immunised at 20° C. produce serum agglutinins, often to a very high titre. If the frogs are transferred to 8° the titre is at once reduced to a very low figure. On their return to 20° it is restored.

3. Experiments suggest that similar phenomena occur in nature in fresh-water fish.

4. Acquisition of the potential for antibody production is thus distinct and separable from the production of antibodies and their appearance in the circulation. In cold-blooded animals the second stage is the more affected by temperature.

5. It is suggested that the techniques described in this work may usefully be employed in the study of more general immunological problems.

I wish to express my thanks to the Royal Society for a grant in aid of this work.

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# A NEW MYCOBACTERIAL INFECTION IN MAN

## I. CLINICAL ASPECTS

P. MACCALLUM

## II. EXPERIMENTAL INVESTIGATIONS IN LABORATORY ANIMALS

JEAN C. TOLHURST and GLEN BUCKLE

## III. PATHOLOGY OF THE EXPERIMENTAL LESIONS IN THE RAT

H. A. SISSONS

## IV. CULTIVATION OF THE NEW MYCOBACTERIUM

GLEN BUCKLE and JEAN C. TOLHURST

*From the Pathology Departments of the Melbourne University  
and Alfred Hospital, Melbourne*

(PLATES XXII-XXIX)

## I. CLINICAL ASPECTS

P. MACCALLUM

ON examination of microscopic sections of a biopsy specimen (29/6/40) from the margin of an ulcer in the skin of the leg of a boy aged  $2\frac{1}{2}$  years, the tissues were found to be teeming with acid-fast bacilli. The child was a patient at a private hospital clinic in the country district of Bairnsdale in Victoria, and since the case was observed 4 additional instances of skin ulceration with similar invasion by acid-fast bacilli have been seen in the same clinic. Recently another example in a patient in the Colac district, over 200 miles from Bairnsdale, has been investigated. It happened that the second Bairnsdale patient was admitted to hospital while the first was still under treatment there. The clinical characteristics of the ulceration in this case (a girl 16 years of age) were so like those of the first as to lead the clinician, in sending a biopsy specimen, to call attention to the similarity. This was found to extend to the histological picture and the bacteriological findings. Very shortly afterwards the third patient (a married woman 51 years



of age) came to the same hospital for treatment of an ulcer and again the physician's diagnosis accurately anticipated the biopsy report. The two other Bairnsdale patients, a boy of 13 years and a single woman of 45, appeared three and five years later at the same clinic.

### *General considerations*

Clinically the ulcers were first thought to be tuberculous, a diagnosis which the finding of acid-fast bacilli appeared to confirm. The organisms were in such abundance (fig. 2) that their presence was obvious even with Gram-staining, which first revealed them as diphtheroid forms in histological sections. The abundance and grouping of the bacilli and the absence of the common histological pattern of tubercle, however, cast some doubt on this diagnosis from the beginning. The report on the first biopsy specimen drew attention to the absence of this cellular reaction and to the distribution of the bacteria in large numbers in phagocytes, recalling that of the *Mycobacterium lepræ* sometimes seen in skin leprosy.

Material from the first three cases was examined bacteriologically in Professor H. A. Woodruff's laboratory with special attention because of the histological appearances. When it was found that the organisms, in spite of their abundance in the tissues and exudate, failed to grow on any of the media commonly used for cultivation of the tubercle bacillus, and that glandular or other lesions did not result from guinea-pig inoculation, the suspicion that the organism was unusual and causally related to the ulcers was strengthened.

Clinical and other local inquiries failed to give any support to leprosy as a possible diagnosis, but the evidence so obtained was almost equally strong against a too-ready assumption that the condition was tuberculous. Though on clinical grounds the lesions gave no support to the idea that the organism was *Mycobacterium lepræ*, careful inquiry was made into this possibility and Dr R. G. Cochrane of the Lady Willingdon Leprosy Sanatorium, Chingleput, South India, was in no doubt, from his examination of one of the cases (S. F.), that this diagnosis could be excluded. Brigadier N. H. Fairley of the London School of Tropical Medicine gave a like opinion in the fourth case (R. T.).

The Bairnsdale district is a mixed farming one. Inquiry about a possible animal reservoir gave no immediate lead to further investigation along this line. Rats are common in the district, but none of the periodic rat or mouse "plagues" had occurred recently nor had any epidemic disease been noted in rats. The patients lived in good home conditions at widely separated points, five of them in or around the town of Bairnsdale, and were strangers to one another. The sixth patient was from Colac, also a dairying district, over 200 miles away. The range of age and occupation provided no evidence of common factors of contact nor ready pointers to a

common source of the inoculum which the histories of superficial abrasions suggested. No seasonal association was traceable in the histories. Some investigation of soil is being made.

With the possible exception of the first case, where the "bronchiectasis" of the mother, in spite of negative sputum examinations, might have been tuberculous, none of the information obtained from the personal or family histories or the X-ray and other examinations of any of the cases was at all suggestive of tuberculosis.

When the condition was diagnosed in the third patient (Mrs S. F.), it was arranged that she should be transferred to a city hospital for further investigation and the same arrangement was made when the fourth case (R. T.) appeared three years later. Tolhurst and Buckle took up the further bacteriological investigation of these two cases and of the sixth case. No success attended any of their attempts to cultivate the acid-fast organism directly from the tissues of the first four patients. Growth in the first instance was obtained only after passage through rats, but, as the result of experience gained with organisms so established, direct cultivation from the ulcer was successful in the sixth patient.

#### Case histories

The lesions in these six cases were solitary and the ulcers initially small and single, though further breaches of surface in the immediate neighbourhood occurred in some as the lesion developed. In the first four the site of ulceration was between knee and ankle and in the last two on the forearm, one of them near the wrist. The beginnings of the ulcer were ascribed by the patients to trivial breaches of the surface from various causes—friction of a rubber boot, slight abrasion from a fall on a stair, an insect bite—or no initiating cause was assigned.

**Case 1.** J. E., a male aged 2½ years, following an abrasion on the lower anterior surface of the left leg, which at first appeared to be healing quickly, developed after a few days a small slough of match-head size in the middle of an indurated inflamed area ("cellulitis") extending over some two-thirds of the outer half of the leg above the abrasion. This, after fomentation and incision for suspected pus, became an ulcer with irregular undermined edges. No pus was found. On incision the fat felt like scirrhous carcinomatous tissue under the knife. Abundant acid-fast bacilli were found in biopsy sections and *Proteus vulgaris* and scanty streptococci were grown. In a period of six months this ulcer denuded the limb laterally of skin and subcutaneous tissue down to the muscle over more than half the leg surface. It had a sloughing base and a hard, sharply cut, irregular edge and became foul smelling. For the first two months the child remained remarkably well and no temperature rise was recorded. The condition was thought to be tuberculous notwithstanding the anomalous biopsy findings, both at a country hospital and at the city hospital for children to which he was transferred in the fifth month. The child's condition deteriorated before transfer, despite general and a variety of local measures, and he ran a temperature of 99-101° F. while in the city hospital. Examination of two inguinal lymph glands gave no evidence of tuberculosis. A fortnight after admission, while undergoing heliotherapy, he died within 24 hours of the onset of an acute abdominal condition which post-mortem examination did not explain.

There had been no known contact with a tuberculous subject, but the mother, who died of pneumonia and meningitis said to be pneumococcal, had been treated for bronchiectasis for years. Repeated examinations of the mother for tuberculosis were negative, while X-ray examinations of the child's chest showed no lesion. Negative at two months, the leg, after 5 months showed some periosteal reaction in both bones. Mantoux test: 1:10,000 negative, 1:1000 positive. Tuberculin patch test positive.

**Case 2.** B. S., a domestic servant aged 16, single, had a persisting scab on a slight abrasion of the upper part of the right shin from a fall on a stair. Five months later she reported with a pin-head hole discharging yellow pus. While under treatment with elastoplast, inflammation extended down the shin. Three months later incision released much pus and, after "glove" drainage, a large slough separated, leaving a sharp-margined ulcer with undermined edges and a "wash-leather" base. Biopsy section showed abundant acid-fast bacilli and a gross mixed infection. Six weeks later an area 6×4 inches including the undermined margin was excised, diathermy applied to the cut edges and a track cut round the ulcer about half-an-inch beyond its margin. Two further ulcers about one inch in diameter developed from infiltrations appearing beyond the ulcer margin above the external malleolus. Two similar infiltrations nearby did not break down. The lesion ceased to extend, pinch skin grafts were applied when the area looked clean, and four months later the patient was discharged with the surface healed. The total duration was 18 months. X-ray examination of the chest showed no lesions. No tuberculosis contacts were traced. The Wassermann test was negative.

**Case 3.** S. F., a farmer's wife aged 51. A flat, slightly irritable reddish area the size of a small pea on the lower third of the left shin became after two weeks a small pin-head sore, which she treated with ointment and bandaged. Between four and five months later she saw a doctor, and after six weeks of elastoplast treatment the ulcer had become about one inch in diameter. It had a deep sloughing base, with raised and indurated edges, and it was surrounded by inflamed skin. At six months she was transferred to a city hospital with a reddish indurated area 4 inches in diameter enclosing two ulcers separated by a bridge of skin. Sections showed abundant acid-fast bacilli in the tissues and *Staphylococcus aureus* and an unidentified diphtheroid were grown from the discharge. After three months' treatment, during which the ulceration fluctuated but responded slowly to elastic sponge pressure dressing, the patient was returned to the country hospital with the ulcer healing. Continuation of this treatment led to complete healing in two months. The total duration was 17 months. X-ray examination of the lungs and tibia showed no lesions. Mantoux tests were positive (human and bovine), reaction to "Johnin" doubtful. The Wassermann was negative.

**Case 4.** R. T., a male aged 13. A small, non-tender lump on the upper part of the left shin, definitely stated to have followed an insect bite, broke down and formed a scab after two or three days. A small deep hole treated at home for about six weeks showed, on reporting to hospital, a reddish edge, a surrounding bluish indurated area and a slight yellow discharge. Incision, with later excision of the edges (fig. 2), was followed by extension of the ulcer. Transfer to a city hospital followed, but despite general measures, splinting and a variety of dressings and local treatments, the inflammation extended up and down the leg. *Pseudomonas pyocyanca*, *Staphylococcus aureus* and *Streptococcus viridans* were grown from the wound. Incision of an indurated area extending almost from knee to ankle released much pus, but progress was not arrested until, after the separation of sloughs, ulceration had involved two-fifths of the leg surface and exposed the tendo achilles. The patient was well till the infection spread, when, for a few days, temperatures as high as 102° were recorded. Acid-fast bacilli were still numerous in smears from the ulcer when the patient

elected to return to hospital at Bairnsdale. Here, after further treatment and skin-grafting, healing was complete after a further 7 months. The total duration was 15-16 months. There was no known contact with tuberculosis. X-ray examination of the chest showed no lesions. The Wassermann test was negative.

Case 5. Miss C., aged 45, engaged in household duties, noticed a small, slightly irritable, whitish-centred, raised subcutaneous induration "like a bull-ant bite or little blind boil" on the anterior inner surface of the left forearm just above the wrist. The indurated area slowly reached a diameter of about 1 inch before the centre broke down, and about 6 weeks from the first observation the patient sought treatment for an ulcer about  $1\frac{1}{2}$  inches in diameter with undermined edges. Diagnosis was made on clinical appearance ("like an ulcerated epithelioma") and on the presence of abundant acid-fast bacilli in smear preparations. Biopsy was not done. At Bairnsdale, an attempt at culture on Dorset's egg medium gave an apparent growth which was probably due to the abundance of acid-fast bacilli in the inoculum, as subculture did not succeed. Cultures of *Proteus* were obtained. Treatment was ineffective before excision and skin-grafting. The "pinch" grafts were in no wise hindered in their rate of growth by the presence of the acid-fast bacilli, which persisted in smears from the surface between the grafts almost up to the stage of their coalescence.

Case 6. K. R., aged 26, a male dairy-farm worker. A slightly irritable, angry-looking but painless ulcer near the middle of the outer surface of the left forearm began as a small pimple. This during three weeks developed a purplish centre which gave place to a central yellow slough half-an-inch in diameter, surrounded by inflamed oedematous skin fully 2 inches across. The adherent, "dry chamois-leather-like" area of slough more than doubled in a week and induration was present almost from wrist to elbow (fig. 1). Despite sulphadiazine and penicillin and after some apparent initial improvement, the ulcer extended. Incision released pus. Abundant acid-fast bacilli and staphylococci were seen in smears and *Staphylococcus aureus* was grown. The area was excised and skin-grafting operations were successful at the third attempt. The total duration was 4 months. No disturbance of temperature while in hospital, save immediately following operation ( $99^{\circ}$  F.). Polymorphonuclear leucocytosis up to 19,400. No regional lymph-node enlargement or tenderness. Wassermann and Kahn tests negative. No evidence of tuberculosis in family history or from chest X-ray. No skin trouble during war service in Pacific Islands.

The four adult Bairnsdale patients, when examined in April 1947, had all maintained good health; there had been no breakdown nor recurrence of the infection at the site of the healed lesions and no evidence of the appearance of similar infection elsewhere. In the case of Mrs S. F., the only patient whose lesion healed without skin-grafting, the skin at the site had some brownish discolouration and was slightly scaly. The area was depressed and there had been no regeneration of subcutaneous fat. The skin however was flexible and the amount of scarring negligible.

### *Gross characters of the ulceration*

The ulcers were thus characterised by indolent extension from the centre of a small breach in a solitary area of inconspicuous, slightly irritable induration; by intractability to treatment; by pyogenic reaction and oedema and often by acceleration and aggravation of the condition by the therapeutic measures taken; by sloughing and denudation of large areas with little or no disturbance of health in the

absence of complications. Spread was by extension (often eccentric) of marginal induration, with breakdown resulting in steep-sided or even undercut edges of scalloped outline exposing necrotic-looking tissue on floor and walls. Occasionally the subcutaneous spread resulted in nodules of infiltration and even areas of ulceration beyond the margin of the main lesion. Apparent healing at one part of the margin might accompany focal necrosis with congestion at another. Loss of resistance to probing was a better gauge of the progress of destruction than surface inspection. A striking feature after the separation of the sloughs and as the ulcer extended was the development from the exposed fascia of an abundant gelatinous mass "like blubbery granulation tissue", which could be readily wiped off with gauze. Its histological characters were not determined.

### *Histology*

Except in the fifth case, where the diagnosis was made on the clinical findings together with the demonstration of abundant acid-fast bacilli in a smear of material from the base of the ulcer, histological examination of tissue excised from the margins or base of the ulcers was made on several occasions. Certain features were common to all. The tissue lining the ulcer wall and floor was necrotic, necrosis being most extensive in the fatty tissue, in which it extended beneath the dermis for varied distances. The obscuring of the outlines of the affected fat cells resulted in a smeary appearance of the tissue. Fascia removed from an ulcer base in one case (B.S.) was not affected to the same extent, but was swollen and showed some infiltration with leucocytes. In and about these areas tenaciously acid-fast bacilli were found in enormous numbers, in every instance grouped characteristically in sharply defined oval or rounded masses often as great in diameter as fat cells, as well as in smaller groups and scattered units.

The appearances were suggestive of enclosure of the bacteria in distended phagocytes, though nuclei often could not be seen. The bacilli were also found in smaller numbers, both free and in phagocytes, in the œdematous connective tissue about the blood vessels in the surrounding fat, in the overlying dermis and about hair follicles and sweat glands.

The blood-vascular engorgement, œdema and polymorph emigration about the necrotic focus varied from specimen to specimen but appeared to be correlated with the degree of pyogenic infection. In the indolent phase the cellular accumulation was mainly of macrophages, together with scattered lymphocytes and plasma cells. Polymorphonuclear leucocytes were scanty except near the ulcer surface, where it was usually possible to demonstrate bacteria. In other stages of the disease more active inflammation of pyogenic type was seen, where vascular engorgement and œdema were marked, polymorphs

were numerous and in some cases typical granulation tissue had developed, particularly near the ulcer margin. In some cases a few Gram-positive organisms could be demonstrated in the surface zone; in others a gross mixed infection with staphylococci, streptococci, Gram-negative bacilli and Gram-positive square-ended bacilli was present.

No sign of tubercle follicles, giant cells, endothelioid grouping or caseation was seen in any of the sections examined. Though the filling of the phagocytes in the subcutaneous tissue with acid-fast bacilli recalled the macrophage reaction seen in this situation in leprosy, there is of course nothing in this phenomenon diagnostic of that disease, and such appearances are common enough in certain circumstances in tuberculous infections. No involvement of nerve trunks was observed.

### *Discussion*

Though the evidence of the causal relationship of the mycobacterium to the ulceration in the human being is incomplete in that no attempt has been made to reproduce the lesion in man by inoculation, the direct evidence is very strong. The mycobacterium is constantly present in lesions of closely similar clinical behaviour in a series of patients. It is the only organism invariably present. Quantitatively its distribution in the lesions is directly related to the focal damage. The tissue response, particularly of the phagocytes, to the multiplication of the organisms is one characteristic of mycobacterial infections in certain circumstances.

The further investigations described in the following sections of this paper also lend strong support to the suggestion of causal relationship, and (apart from clinical objections) render improbable the alternative hypothesis that the lesions is a form of symbiotic gangrene and the acid-fast bacillus a contaminant without aetiological significance or at most a participant in a symbiotic association. The other flora found when ulceration had occurred, varied. To these can be ascribed the more acute pyogenic inflammatory process, with gross subcutaneous oedema, extending from the ulcer margin in the course of treatment and accompanied by systemic febrile reactions. This infection no doubt plays a part in determining the extent of tissue destruction.

The difficulty of cultivation of the present organism and its behaviour towards laboratory animals raises the question of its relationship to other mycobacteria, especially those of leprosy and tuberculosis. The only lesion in a human being recorded as due to rat leprosy is that of Marchoux (1923). The description does not correspond in any respect to the cases here recorded and the response of his rats to inoculation was different from that observed by us. Of more direct interest are the observations of Cilento (1942), who in his discussion of leprosy in Australia states, "during the course of

the last few years there have been several patients referred for examination from the Rockhampton area who have shown a single tiny ulcer without anæsthesia. From the depths of these ulcers organisms indistinguishable from *Mycobacterium lepræ* have been obtained. Cultivation experimentally has excluded tubercle bacilli in these cases. (A tentative diagnosis of Bazin's disease had been made though this was only a doubtful possibility) ”.

An acid-fast soil contaminant was clinically improbable and syphilis could be excluded. Since leprosy is endemic “to a small extent among white persons and others” in Queensland, Cilento discussed the possibility of the ulcers being a local variant in biological response to the *Mycobacterium lepræ* and cited Ryrie's observations on vitamin-A lack and acute leprous ulcers in Malaya and leprous alopecia in Japan and China. He points out that “no such single ulcer manifestation as a sole sign of leprosy has been recorded, so far as is known, anywhere in the world, so that the probabilities are against these cases being leprous”. He quotes the enquiry of the *International Journal of Leprosy* into the admittedly short-term results of removal of isolated nodules by Wayson and others in cases of leprosy without demonstrable systemic involvement, and records the apparent success of wide excision in the treatment of one of the Rockhampton ulcers. He thinks the indolence and intractability of the ulcers, if leprous, can possibly be ascribed to a high ratio of resistance existing in the white population as a result of excellent living conditions. Though discussing them as possibly due to leprosy, he concludes that for the present these lesions are undiagnosed.

The Queensland story of finding in the depths of ulcers described as tiny, single, without anæsthesia and clinically indolent and intractable, of acid-fast bacilli not identifiable culturally or experimentally as tubercle bacilli is so similar to the Victorian one as to provoke some expectation of identity in the causal agent.

While leprosy is not known to assume this ulcerative form, there are clinical manifestations of tuberculosis which are virtually indistinguishable clinically from those met with in these patients. In searching for records of similar cases, an instance of such intractable ulceration following an abrasion by a garden stake of the left leg of a girl of  $7\frac{1}{2}$  years was found. There was extensive loss of skin and amputation was considered. The only difference in the clinical history was the appearance later of other ulcers on the right leg and left forearm. The ulcers swarmed with acid-fast organisms which proved to be tubercle bacilli. Ultimately healing followed excisions and skin-grafting. In the Bairnsdale cases no other similar lesions or nodular infiltrations had appeared in any of the patients while under treatment, nor in any of the four surviving patients has there been any sign of recurrence, locally or elsewhere.

The very success which eventually attended the attempts to

cultivate the present organism would appear to confirm the clinical exclusion of leprosy. While the clinical distinction from tuberculosis is not so clear-cut, the failure attending the usual laboratory procedures for its demonstration excludes this diagnosis also, and the close clinical similarity to certain cases of tuberculous infection of the skin in man is in striking contrast with the observations of Tolhurst and Buckle on the behaviour of the Bairnsdale organism in animals. Their investigations on the peculiarities of the pathogenicity of the new *Mycobacterium* for animals and on the conditions for its optimal growth, particularly those of temperature and moisture, exclude *Mycobacterium tuberculosis* and any hypothesis that such modification of characters can come within the range of meaning of the term attenuation.

The investigations described in later sections of this communication provide us with valuable new means of comparative examination of the mycobacterial group. A mycobacterial infection cannot be dismissed as aetiologically unimportant on the ground of failure of cultivation in ordinary laboratory conditions or of the guinea-pig test. Moreover the presence of "diphtheroid bacilli" in a section of tissue should always suggest the advisability of making a Ziehl-Neelsen preparation. It is also insufficient to assume a tuberculous infection from the presence of acid-fast bacilli, or, in an area of endemic leprosy, to make a diagnosis of leprosy without further differential investigation on the lines now indicated.

Rich (1944) points out that the tubercle bacillus introduced into a normal non-sensitised person will show the phenomenon of multiplication within macrophages with little or no destruction of tissue and with no general constitutional disturbance. After a period of some weeks, with the development of sensitivity, both phagocytic and constitutional complacency disappear, and the intra- and extra-cellular response changes. On the basis of a common type and range of reaction to the pathogenic mycobacteria in man and animals, the tissue response seen in these patients is in keeping with the hypothesis of a first experience with a new mycobacterial infecting agent in which the development of secondary reactivity of the sensitisation type is very slow to develop. Indeed we have no good evidence in these cases that it does develop, though recent success with the cultivation of the organism, particularly on fluid media, may make some test of this possible. Clinically it is masked by the complication of other types of infection. The demonstration of properties in common with tuberculous infection may yet lend interest to the finding of a positive Mantoux reaction in the child of 2½ years. Unfortunately there is no record of a negative stage in this patient. On this hypothesis also it is less probable that the skin infection has an endogenous source; the histories and the occurrence of the ulcers on exposed parts suggest inoculation rather than localisation by trauma. The source of the infection remains unexplained.



Some points of interest emerge from the story of the treatment of these cases. One is that fomentation, such applications as elastoplast and most active measures result in aggravation of the condition. Of the dressings and lotions applied, the simplest were the least disturbing—e.g. saline. The only effective measures were wide excision, followed, when vigorous granulations were present, by skin-grafting and centripetal elastic sponge pressure and simple lotions applied with judgment in accordance with the principle advocated by Mr F. A. Maclure in the treatment of leg ulcers, namely that of approximating as nearly as possible to the conditions of elastic resilience normal for fluid exchange in the subcutaneous tissue. It is perhaps significant that skin-grafting implies the application of similar pressure. It is also of interest that, after the development of vigorous granulations, the presence of numerous mycobacteria in the surface exudate in no way hinders the success of skin-grafting, an observation in keeping with the general experience of plastic surgeons in relation to other bacteria, which in such circumstances cease to be effectively pathogenic.

## II. EXPERIMENTAL INVESTIGATIONS IN LABORATORY ANIMALS

JEAN C. TOLHURST and GLEN BUCKLE

In April 1941 the third patient in this series, Mrs S. F., was admitted to the Alfred Hospital. The discharge from her ulcer was purulent but not copious, and smears revealed many acid-fast bacilli resembling tubercle bacilli except that they occurred in masses or bundles as well as singly. *Staphylococcus aureus* and diphtheroid bacilli were also present in smears and in routine aerobic and anaerobic cultures.

Saline washings of the pus were treated variously with sulphuric acid, sodium hydroxide or gentian violet to destroy the pyogenic organisms and cultures were made on blood agar, Petragnani's medium and Twort's medium; these yielded no growth of acid-fast bacilli, although some tubes were kept for over a year.

A guinea-pig injected subcutaneously with ground-up tissue and pus, untreated, did not develop tuberculosis, and no evidence of avian tuberculosis was discovered in mice inoculated subcutaneously. A rat so inoculated died after 13 months, and at the site of injection in the inguinal region a lump the size of a small pea was found to contain acid-fast bacilli; no other lesions were found. An attempt to produce infection in another rat by subcutaneous inoculation with this material failed. A rat inoculated intraperitoneally with pus from the patient's ulcer died after 4 months; the only apparent abnormality was in the lungs, smears from which showed many kinds of bacteria, including acid-fast bacilli; no sections were made.

A saline extract of lung tissue was injected intraperitoneally into another rat, which after 9 months became visibly distended and was killed. About 50 ml. of fluid were recovered, loaded with acid-fast bacilli. This was wholly unexpected. We could find no record in the literature of a similar observation. Moreover the material used to inoculate this rat was rat-lung tissue obviously infected with a variety of bacteria, and although we had frequently seen similar lesions in old rats we had never before examined them for acid-fast bacilli. It was therefore uncertain whether the acid-fast bacillus associated with the ascites was really from the patient S.F. or merely an extraneous invader. It was decided to inoculate another rat intraperitoneally with the ascitic fluid. This rat developed ascites within 4 months and the fluid was injected intraperitoneally into a fourth rat. Meanwhile attempts to cultivate the bacillus from the fluid had failed. The fourth rat was observed for several months and then forgotten: 16 months from the time of inoculation it was found that the limbs were swollen, oedematous and ulcerated, and that the tail had sloughed off (fig. 3). The ulcers were crowded with acid-fast bacilli and it was evident that we were dealing with an organism capable of producing ulceration of the skin, and possibly originating from the human ulcer. From this ulcerated rat a series of passage inoculations has maintained the acid-fast organism for nearly four years.

In March 1944 the fourth patient, R.T., was admitted to the Alfred Hospital. Smears from his ulcer contained many acid-fast bacilli, and Gram-positive cocci and Gram-negative bacilli which were identified in culture as *Staph. aureus*, *Strep. viridans* and *Ps. pyocyanea*. Saline suspensions of the purulent discharge were treated with sulphuric acid and used to inoculate a great variety of media, but although the cultures were kept for many months no growth was observed. A guinea-pig inoculated with untreated pus showed no evidence of tuberculosis when killed 6 weeks later. Untreated pus was injected intraperitoneally into male white rats which, after a period of 3-7 months, developed ascites, the fluid being loaded with acid-fast bacilli.

These observations and the similarity of the lesions in the patients made us confident that we had two strains of the same bacillus, and as both strains were now growing in rats, we were able to make a detailed comparison. The similarity of the lesions in animals and of the morphological and cultural characters of the strains has since confirmed our opinion that they are the same species. The strain R.T. has been maintained for over 3 years by a series of passage inoculations in rats.

#### *Pathogenicity*

In view of the great interest of the initial observations, the pathogenicity of the bacillus was more thoroughly investigated. This report relates particularly to the study of 76 white rats, 16 white

mice, 12 guinea-pigs, 2 rabbits, 1 fowl and 3 lizards. All these species were inoculated with strain R.T., while strain S.F. was studied in rats, mice and guinea-pigs only. The recently found strain K.R. (case 6, Colac) was inoculated into a few rats, mice and guinea-pigs.

### Rats

*Intraperitoneal inoculation in male rats.* Intraperitoneal inoculation of 52 male rats with either pus from the patient's ulcer (strains R.T. and K.R.) or fluid from another rat (strains S.F. and R.T.) led to ascites in 49, the remaining animals being killed early, before ascites had developed. The first sign of disease was swelling of the scrotum with apparent fixation of the testes in the scrotum, which occurred at any time from 2 to 9 months, though usually after 4 or 5 months (fig. 4). The rate of swelling of the abdomen varied greatly, so that the animal might be enormously distended 2 weeks after swelling of the scrotum was first observed, or it might swell gradually over a period of 3 months (fig. 5). Gross subcutaneous œdema of the body wall was unusual. Sometimes the animals died without any outward signs of disease other than a swollen scrotum and abdomen; frequently they were killed at this stage to obtain fresh uncontaminated material for cultural or animal experiments. In rats with ascites which survived this stage of the disease, ulceration of the scrotum occurred sooner or later, commencing as a small red area which rapidly developed a hard black crust. Subsequently, perforation of the scrotal wall occurred, with escape of the fluid. Some rats were observed biting the ulcer. In some cases ulceration was so extensive as to expose the testes. Healing of the ulcer now proceeded. After a month or two œdema of the tail or feet and rarely of the face was observed and was followed by ulceration (fig. 6). The duration of the disease from the time of inoculation until death was about 12 months. During the last weeks of life the animals appeared sick and miserable, but it was observed that before this, in spite of having suffered from cutaneous œdema and ulceration for many months, they usually remained in good general condition with bright eyes and healthy coats. Alopecia was looked for, and in some animals thinning of the hair was seen, but it was not different from that observed in a control group of rats kept under the same conditions. In a few rats complete hairlessness was observed in small areas adjacent to ulcers, as on the back near the root of the tail.

In rats with ascites the volume of fluid varied from 0.5 to 200 ml.; it was yellow and turbid and clotted readily. It contained many inflammatory cells, chiefly large mononuclear phagocytic cells, many of which contained acid-fast bacilli varying from one or two to a mass of hundreds filling a cell (figs. 9 and 10). The epididymis commonly showed at one or both ends conspicuous white lesions 0.5-5 mm. in diameter and whitish lesions were also present on

A NEW MYCOBACTERIAL INFECTION IN MAN



FIG. 1.—Ulcer on the forearm of case 6 (K.R.) photographed the day before excision.

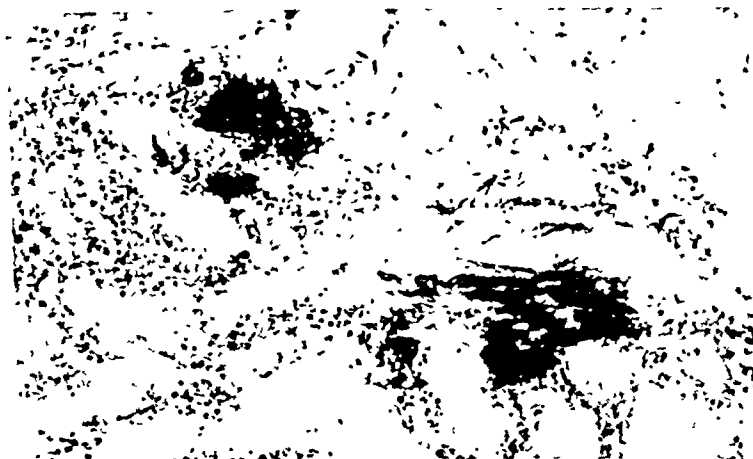


FIG. 2.—Low-power view of section of skin from case 4 (R.T.). The two large dark irregular masses are collections of acid-fast bacilli. Ziehl-Neelsen and hæmatoxylin.  $\times 160$ .



FIG. 3.—Rear view of rat showing oedematous ulcerated hind limbs and ulcer at site of lost tail: 16 months after intraperitoneal inoculation.



A NEW MYCOBACTERIAL INFECTION IN MAN



FIG. 4.—Right : normal male rat. Left : rat with swollen scrotum about 4 months after intraperitoneal inoculation.



FIG. 5.—Rat with swollen scrotum and abdomen about 6 months after intraperitoneal inoculation.



A NEW MYCOBACTERIAL INFECTION IN MAN

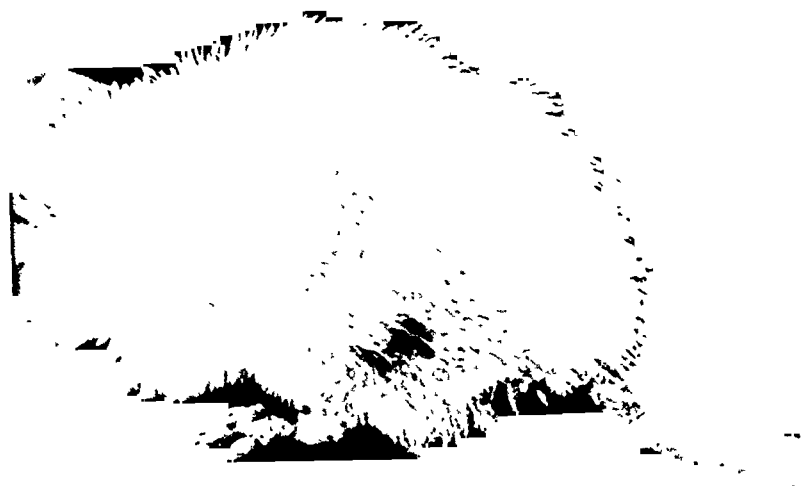


FIG. 6.—Male rat showing oedema and ulceration of tail and limbs 13 months after intraperitoneal inoculation. (The third rat in a series originating with the intraperitoneal injection of a fifth-generation culture of strain R.T.)

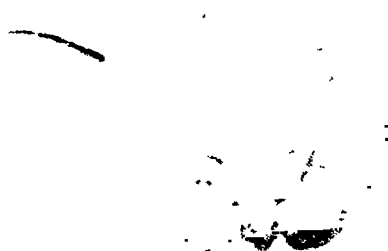


FIG. 7.—Male mouse showing gross oedema of the subcutaneous tissues 4 months after intraperitoneal inoculation.

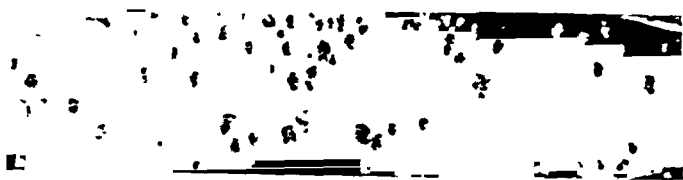


FIG. 8.—Colonies of strain K.R. on Petraghani's medium : primary culture 9 weeks old.  $\times 1.3$ .





A NEW MYCOBACTERIAL INFECTION IN MAN

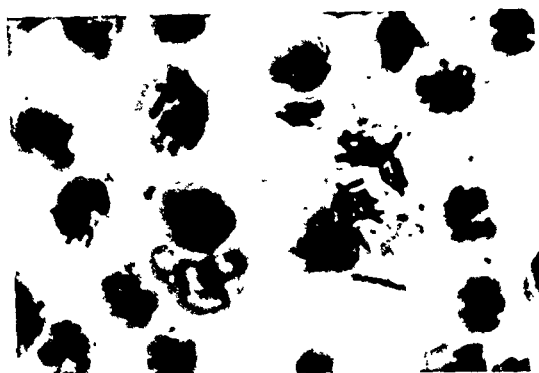


FIG. 9.—Smear of peritoneal fluid of male rat showing small clumps of bacilli within a cell. Ziehl-Neelsen and methylene blue.  $\times 1250$ .

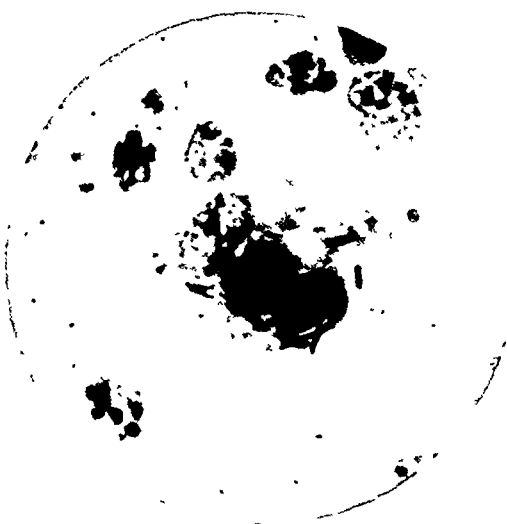


FIG. 10.—Smear of peritoneal fluid of male rat showing two large clumps of bacilli within a cell. Ziehl-Neelsen and methylene blue.  $\times 1250$ .

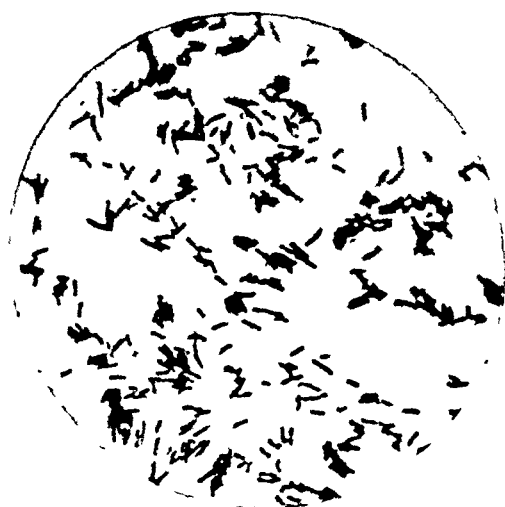


FIG. 11.—Smear of culture of strain S.F. on Petraghani's medium. Ziehl-Neelsen and methylene blue.  $\times 1250$ .



adjacent parts of the peritoneal surface of the scrotum. Both epididymal and scrotal lesions contained acid-fast bacilli in large masses. There were often patches of white fibrinous deposit on the surface of the peritoneal organs and minute grain-like lesions were occasionally seen in the omentum. Post-mortem examination of rats in which the scrotal wall had been ulcerated and perforated showed no macroscopic peritoneal lesions. The scrotal ulcer had often healed, leaving a scar.

In the thorax a small amount of yellow fluid was sometimes found, and this contained cells but usually no organisms. When subcutaneous oedema was present the fluid in the tissues was usually clear and colourless and contained only very occasional cells and bacilli. The ulcers of the oedematous tail or feet varied in depth and contained on the surface pyogenic bacteria as well as acid-fast bacilli. In the lungs no lesions associated with acid-fast bacilli were encountered and microscopical examination revealed a few isolated bacilli on one occasion only. Hence the initial observation of acid-fast bacilli in the lungs of the first rat inoculated intraperitoneally was unusual.

*Intraperitoneal inoculation in female rats.* Ten female rats were inoculated by the intraperitoneal route. Swelling of the abdomen was never observed, but oedema of the limbs or tail occurred in three rats and was accompanied by ulceration in two of them. The lesions, which became evident after about 7 months, were similar to those seen in male rats.

*Intracerebral inoculation.* One female rat was injected intracerebrally. When killed after 8 months it presented typical ulcerated lesions of the tail and left hind foot, while the tissues of the snout were oedematous.

*Subcutaneous inoculation.* This was carried out in 13 rats. Five developed an ulcer at the site of inoculation, one after 7 months, two after 10 months, one after 14 months and one after 20 months. A sixth which died after 13 months had a small lump at the site of inoculation. In the other seven the inoculum was apparently absorbed, for no lesions developed although two rats survived for nearly 12 months, one for 14 months and one for 20 months. Possibly the location of the subcutaneous injection influences the development of a lesion. Apart from local ulcers no macroscopic lesions were found *post mortem*.

*Control rats.* Four rats inoculated with autoclaved butter-fat were maintained as controls in tins kept adjacent to the experimental rats. One was killed after 4 months and one after 20 months; one died after 18 months and another after 22 months. No evidence of infection with the acid-fast bacillus was found in any of them.

Six months after inoculation, one experimental rat gave birth to three young. At this time the mother had no obvious lesions, although two months later she developed oedema of the tail, which subsequently ulcerated. The young rats were removed from the parent a few weeks after birth and two of them were kept for over a year without any sign of infection.

#### Mice

In five mice an infection with acid-fast bacilli was established by the inoculation of pus from the ulcer of R.T., and five others were infected with fluid from other mice or rats. These ten mice were males, eight of which were inoculated intraperitoneally and two

subcutaneously. In all, the course of the disease and the post-mortem findings were similar.

Three to six months after inoculation, swelling of the subcutaneous tissues and abdomen commenced. Whereas in rats gross œdema of the subcutaneous tissues of the body was rare, in mice it was common and marked. The œdema involved the subcutaneous tissues of the whole body and head so that the animals were two or three times their normal size and presented a curious appearance (fig. 7), although until just before death they were bright and lively. These mice usually died in 4-6 months, the period between the first sign of swelling and death ranging from 10 to 40 days. One mouse developed a swollen ulcerated hind limb and an ulcer of the scrotum, another an ulcer of the scrotum. A small area of complete alopecia was observed on the body around the base of the tail in two other animals.

The subcutaneous tissues of the body were usually distended with clear colourless fluid and as much as 8 ml. was recovered on one occasion. The peritoneal cavity usually contained from 0.5 to 5.0 ml. of turbid fluid, and a little fluid was found in the thorax. The peritoneal wall appeared normal and there was no fibrinous exudate on the organs. Macroscopic lesions of the epididymis were recorded only once, but microscopic lesions similar to those in rats were found in 7 out of 8 mice in which sections were cut. The subcutaneous tissue fluid contained very occasional inflammatory cells and bacilli. In the peritoneal fluid, cells and organisms were more numerous, but not nearly as plentiful as in rats. On one occasion a few bacilli were found in thoracic fluid.

Male mice inoculated intraperitoneally with ascitic fluid (strain S.F.) and with material from the human ulcer of K.R. gave lesions similar to those described above. Two female mice were inoculated by the intraperitoneal route with ascitic fluid from a rat (strain R.T.). Both died after about five months with no macroscopic evidence of the infection; no sections were made.

### Other species

*Guinea-pigs.* As stated previously, guinea-pigs inoculated subcutaneously with material from the ulcers of S.F. and R.T. showed no macroscopic evidence of disease when killed six weeks later. Another animal inoculated subcutaneously with saline washings from the ulcer of R.T. died in an emaciated state after six months, but no lesions were found. Nine guinea-pigs inoculated intraperitoneally with human or animal material were observed for periods of from 5 to 15 months:  $4\frac{1}{2}$  months after inoculation with ascitic fluid from a rat, one female developed an ulcer on the nose containing acid-fast bacilli; this healed and no other lesions were found. Two of the remaining animals showed collections of yellowish material containing degenerate acid-fast bacilli in the omentum, with no signs of progressive disease; the other six were apparently unaffected by the injection.

A guinea-pig was injected subcutaneously with tissue from the ulcer of K.R. but no lesions of any kind were found ten weeks later. A male guinea-pig inoculated by the intraperitoneal route with the same material revealed no macroscopic lesions after six months.

*Rabbits.* A male rabbit was inoculated intraperitoneally with a suspension of tissue from the leg of R.T. It died after 15 months, the only lesions attributable to the injection being yellowish areas on each epididymis, smears of which were teeming with acid-fast bacilli.

Another male rabbit inoculated intravenously and intraperitoneally with ascitic fluid from an infected rat died in 10 months and again the only lesions attributable to the injection were in each epididymis, smears from which

revealed many acid-fast bacilli. Sections of the organs of both animals showed no lesions.

*Fowls.* A white leghorn cockerel was inoculated intraperitoneally with untreated material from the ulcer of R.T. It remained well for 13 months, when it was killed and found to be in a healthy condition. No macroscopic lesions were observed and no sections made.

*Lizards.* Three blue-tongued lizards (*Tiliqua scincoides*) were inoculated intraperitoneally with untreated material from the ulcer of R.T. The first died after 10 weeks. Collections of degenerated inoculum containing a few acid-fast bacilli were lying loose on the surface of the liver but nothing abnormal was found in sections of the organs. The other two animals died after 15 and 20 weeks respectively with no obvious evidence of infection; no sections were made.

### Morphology

Morphologically this organism resembles the human tubercle bacillus. Its average length is  $3\ \mu$  but organisms as long as  $6\ \mu$  are common; variations from  $0.75$  to  $12\ \mu$  are seen. Its width varies from about  $0.2$  to  $0.35\ \mu$ . The bacilli have more or less parallel sides and rounded ends and are often curved. An appearance of branching is seen occasionally. No spores or capsules have been found.

The organisms may occur singly or in groups; two may lie at an angle or a number in palisade formation. Chains are not formed. Collections of organisms frequently occupy the mononuclear and occasionally the polymorphonuclear phagocytic cells in the ascitic fluid of infected rats. Such cells often contain several small groups of bacilli, each group with its axis lying in a different direction (figs. 9 and 10). Possibly the bacilli multiply in these cells, since other cells are found in which there are massive bundles of organisms clearly composed of several large groups. These may attain a size of  $12 \times 7\ \mu$  and distend the enclosing cell, appearances which are characteristic of this bacillus. Extracellular organisms occurring singly or in groups and undoubtedly arising from the epididymal lesions are also found in ascitic fluid. Sections of human and animal lesions usually contain large masses of bacilli (figs. 2 and 12).

The bacillus is Gram-positive if the methyl-violet stain is warmed. It stains brilliantly by the Ziehl-Neelsen method (fig. 16) and is strongly acid-fast. Smears were stained for ten minutes with hot carbol fuchsin. When treated for ten minutes with 3 per cent. HCl in 70 per cent. alcohol, most of the organisms were brilliantly stained, although some were paler. After an hour in the acid-alcohol, some bacilli were completely decolourised; others had a refractile appearance but the majority were still well stained. After four hours in the acid, fewer bacilli were visible and after 24 hours most of them were decolourised, so that instead of bacilli being seen in every field they had to be searched for. Some organisms, however, resisted even this long treatment with acid alcohol and remained deeply stained.

Granules in the form of round or oval beads at the ends or in the middle of the bacilli are not infrequently seen in the same smear with

solidly stained organisms. Yegian and Baisden (1942) and Porter and Yegian (1945) have shown that the beading of tubercle bacilli in smears stained by the Ziehl-Neelsen method is largely effected by the staining technique, and that most of the granules disappear if, after treatment with acid alcohol, the smear is immersed in neutral alcohol for a short time. We have found that this applies also to the new bacillus.

### *Discussion*

It is necessary to consider whether the acid-fast bacilli found in the ulcers of the three patients were identical. If the patient S.F. had been the only source of the organism, doubt would have arisen about the origin of the acid-fast bacilli found among other bacteria in the lungs of the first rat injected intraperitoneally, which subsequently produced ascites in another rat. However, there is no doubt that the organisms in the ulcer of R.T. were transferred to animals, since 14 rats and 5 mice were infected directly by various routes. The organism in the ulcer of K.R. was also proved to be pathogenic for rats and mice. That the bacilli from S.F., R.T. and K.R. are the same is shown by their identical morphological and cultural characters and by the similarity in development, distribution and structure of the lesions produced in inoculated animals.

Evidence that this acid-fast bacillus is not one of the well-known mycobacteria follows from a study of the pathogenicity of the genus. Human or bovine tuberculosis, or "vole tuberculosis", was not produced in guinea-pigs by subcutaneous inoculation (animals killed after six weeks and six months) or by intraperitoneal inoculation (one animal observed for 15 months). Avian tuberculosis was not produced in a cockerel by a very large inoculum of pus injected intraperitoneally, although the bird was observed for 13 months. Lesions were not produced in cold-blooded animals (lizards) within several months following intraperitoneal inoculation.

In 1916 and 1919 Traum described subcutaneous nodules in cattle which on section showed necrotic foci containing acid-fast bacilli. Giant cells were present and the tubercles found were histologically indistinguishable from those associated with the tubercle bacillus. The nodules sometimes softened and discharged pus containing calcareous granules. The organisms were thought not to be tubercle bacilli because they were non-infective for guinea-pigs and were refractory to culture. Many workers have since attempted to identify them without success.

The so-called saprophytic mycobacteria are said to be unable to set up by themselves a progressive infection in mammals. However, some of them when injected intraperitoneally into guinea-pigs in large doses, together with a fatty substance such as butter, may produce extensive lesions simulating those of tuberculosis (Rabinowitsch, 1897). Pinner (1935) isolated a number of acid-fast organisms which produced in guinea-pigs non-tuberculous self-healing lesions not transferable in series through animals, and on this basis he grouped them as saprophytes.

Unfortunately the numerous acid-fast bacilli described (Thomson, 1932; Hagan and Levine, 1932; Schwabacher, 1933; Gordon, 1937; and others)

have not been inoculated, as a rule, into rats and mice by the intraperitoneal route. However, as will appear (section IV), the cultural characteristics of the Bairnsdale bacillus are distinctive.

John's bacillus, which causes a chronic disease of cattle characterised by massive infiltration of the intestinal tract, is said not to be infective for adult laboratory animals. Moreover this organism can be cultivated on Twort's medium at 37° C.

The human leprosy bacillus is said not to be infective for laboratory animals.

The rat leprosy bacillus produces a natural disease in rats and occasionally in mice (Krakower and González, 1940), but the lesions of both the natural and experimental infections differ from those produced with the bacillus here described. Rat leprosy occurs in Australia, although probably less than one per cent. of rats are affected. Lowe (1937) summarised Stefansky's original description of the naturally occurring disease in its "glandular" and "musculo-cutaneous" forms. He also stated that 8-10 months after intraperitoneal inoculation the omentum had often become a large solid mass, several grams in weight, consisting of an accumulation of cells crammed with bacilli. Our personal observations on rat leprosy are limited to five rats inoculated intraperitoneally. After about nine months, three had numerous grain-like nodules on the omentum, mesentery and peritoneal surfaces generally, and the mesenteric and inguinal glands were enlarged. The other two had large confluent masses involving the omentum and mesentery, particularly the mesentery of the epididymis. In all the infected animals the peritoneal lesions were teeming with rat leprosy bacilli. No ascites, cedema or ulceration of the skin, either at the site of inoculation or remote from it, was observed in any of these animals, and we have found no reference in the literature to the occurrence of such lesions following intraperitoneal inoculation with rat leprosy bacilli.

With the new bacillus, the epididymis is the preferred site for primary lesions in male rats; in our experiments, following intraperitoneal inoculation, epididymal lesions were found in all 52 animals. These lesions are present before ascites develops; we found them in three rats which died or were killed after a few months and had no ascites and no swelling of the scrotum. On the other hand, of ten female rats inoculated intraperitoneally only three had lesions containing acid-fast bacilli, and a microscopic peritoneal lesion, occurring in the fatty tissue surrounding the ovaries and of doubtful significance, was found in only one. Both males and females developed exactly similar ulcers of the tail and limbs, that is, at points remote from the site of inoculation, as did one female inoculated intracerebrally. The more frequent occurrence of ulcers of the tail and feet in males is presumably due to re-inoculation of these sites from the peritoneal lesions.

Dissemination to remote parts of the body may be by the blood stream or lymph-channels. The organism then appears to spread in the subcutaneous tissues and to penetrate in places to the skin surface, for lesions may be found extending in the tail an inch or more from the point of ulceration. Ulcers of the skin developing after subcutaneous inoculation show signs of localised spread through the subcutaneous tissues. Similar spread in human tissues is reported in section I.



The virulence of the new bacillus is as yet undetermined. The ascitic fluid of rats varies in its bacillary content, but on two occasions Breed smears gave counts of 1.2 and 3.2 million organisms per ml. It was our practice to use an inoculum of between 0.1 and 2.0 ml. As many as eight consecutive passage inoculations have been carried out without attempting to control the number of bacilli injected, and no significant shortening of the course of the disease has been observed. In one rat the subcutaneous inoculum remained apparently quiescent for about 18 months. This raises the question of the incubation period in man.

The sources of human infection with the bacillus are unknown. The district of Bairnsdale is infested with rats. Through the courtesy of the Public Health Department we have received and examined five of these animals without finding any evidence of infection, but one might expect the incidence of the disease to be small. However, the pathogenicity of the organism for injected rats does not prove that this species harbours it in nature. Whatever the reservoir of the organism the method of transfer to man has still to be elucidated.

### III. PATHOLOGY OF THE EXPERIMENTAL LESIONS IN THE RAT

H. A. SISSONS

#### *Material and methods*

This section deals with the microscopic structure of the lesions produced in rats as part of the experimental investigation described above.

Twenty-eight male and 5 female rats were studied after intraperitoneal inoculation, and material is included from another rat, inoculated intracerebrally, which presented comparable lesions. These animals included examples of each of the strains established (S.F., R.T., and K.R.), but the histological characteristics of the lesions were similar in all animals and a common description is applicable to all three strains. In a few rats the local lesions produced by subcutaneous inoculation were studied. The lesions produced in mice and rabbits by intraperitoneal inoculation are comparable to but much less conspicuous than those in the rat. Their detailed histology is not included here.

The animals were carefully dissected as soon as possible after death and were preserved in their entirety in 4 per cent. formaldehyde. All gross lesions were studied microscopically, and blocks from seemingly unaffected organs were also examined in most of the animals. Sections were stained with hæmatoxylin and eosin, carbol-fuchsin and hæmatoxylin, eosin-azure, and Masson's connective-tissue stain.

A technique which deserves note is the use of the eosin-azure stain, either alone or in combination with light hæmatoxylin staining. In the present material it demonstrated the bacilli with great clarity, and was useful also in the identification and study of inflammatory cells. Pagel (1940), using a similar method to demonstrate acid-fast bacilli in experimental tuberculosis in the mouse, showed that the bacilli, which at first stain with the azure component, after being present in the tissues of the animal for about 10 days stain selectively with the eosin although there is no change in their acid-fastness. In the present material, however, azure staining of the bacilli was a common finding, even in lesions of long standing.

*Lesions in relation to the peritoneal surface*

(i) *Epididymis and scrotum.* This part of the peritoneal surface is always more extensively involved than the rest. The lesions appear as white opaque areas on the surface of the fatty tissue of the epididymis and on the parietal peritoneum of the scrotal wall. In early stages only a few small focal areas are found; later, these become confluent and more extensive. As the lesions progress, firm adhesions become established between the epididymis and the scrotal wall, and diffuse oedema of the scrotal tissues and fatty tissue of the epididymis usually appears. When the lesion extends to involve the adjacent scrotal muscle this tissue becomes firmly adherent to the peritoneum and presently forms a hard plaque to which the skin eventually becomes attached. In contrast to the remainder of the scrotal peritoneum, the testicular surface is usually uninvolved, but in one animal an area of testis adjacent to the epididymis was the site of a lesion. Here, bacilli-containing granulation tissue extended to the interstitial connective tissue of the testis.

Microscopically the earliest lesions in the epididymis are seen to consist of a superficial area of hæmorrhagic necrosis of the fatty tissue. In the centre of such a lesion, in addition to extravasated erythrocytes, fibrin, cell debris and pyknotic nuclear fragments, only occasional degenerating polymorphonuclear leucocytes are present, but surrounding the necrotic areas are zones of cellular accumulation where polymorphonuclear leucocytes, lymphocytes and macrophages are seen in large numbers. These cells occasionally accumulate in the neighbourhood of dilated capillaries, and the inflamed tissues show marked oedema.

In the necrotic areas localised accumulations of great numbers of acid-fast bacilli are present. For the most part they are extracellular and arranged in bulky branching bundles (fig. 12). These aggregates, in which the bacilli lie side by side, take on, as a whole, a curved appearance, and are found not only in the epididymis but wherever large numbers of bacilli are present. In the cellular zone a few of the inflammatory cells contain acid-fast bacilli, but most of the bacilli-containing cells are macrophages. Many of them have vacuolated ("foamy") cytoplasm; those which contain many acid-fast bacilli are enlarged but not otherwise abnormal. Giant cells are not found in the lesions. The bacilli within the macrophages have no characteristic orientation, their long axes being arranged independently of each other. Very occasionally a small dense collection of bacilli occupies a localised region of the otherwise empty cytoplasm. These intracellular aggregates are rarely found in sections; they are seen more frequently in smears of peritoneal fluid (figs. 9 and 10). Even here the bacilli are quite irregularly orientated, and neither the "globi" nor the "rosettes" described by Cowdry and Ravold (1938) and Cowdry (1940) in rat leprosy have been observed. There can be

no doubt that the massive extracellular colonies of bacilli arise by local multiplication, but whether the intracellular organisms are actively multiplying or merely phagocytosed and quiescent is not clear.

The lesions, even in their early stages, commonly show thrombosis and eventual obliteration of small blood-vessels in the necrotic areas. As they increase in size the lesions extend to the deeper fatty tissue which is present between the groups of epididymal tubules. In most cases the tubules themselves are not involved and are found intact within otherwise necrotic areas. Occasionally part of a tubule is involved; its epithelium is destroyed and its lumen occupied by a mass of acid-fast bacilli, disintegrating spermatozoa and necrotic inflammatory cells. The cylinder of material occupying the lumen can be distinguished from the surrounding tissue and the outline of the destroyed tubule is thus preserved.

The oldest lesions, replacing almost the entire fatty tissue of the epididymis, show extensive development of vascular granulation tissue in parts remote from the necrotic areas. This tissue contains numerous inflammatory cells, including many macrophages, but it is only in the zone immediately adjacent to the necrotic areas that acid-fast bacilli are found within these cells.

The lesions commencing on the peritoneal surface of the scrotal wall show the same massing of acid-fast bacilli in the necrotic sub-peritoneal tissue, and here also a zone of cellular accumulation separates the necrotic tissue from the surrounding uninvaded but often œdematous tissue. When outward extension has reached the skin surface and ulceration has commenced, there is often a great increase in the number of acid-fast bacilli in the necrotic tissue immediately deep to the ulcerated part.

(ii) *General peritoneal surface.* This is usually covered by a tenacious filmy layer which, after formol fixation, can be detached only with difficulty. It consists of a fibrinous coagulum enmeshing numerous inflammatory cells of various types, including occasional polymorphs, numerous lymphocytes and abundant bulky mononuclear phagocytes or macrophages, whose cytoplasm occasionally contains numerous acid-fast bacilli (fig. 16). These cells are often arranged in small groups surrounded by a zone of lymphocytes. As shown in fig. 16 the deposited material lies superficial to the splenic capsule, which is not invaded, and the fibrinous layer with its cellular aggregates is quite avascular.

The ascitic fluid contains large numbers of cells similar to those of the surface deposit. Lymphocytes and polymorphs can be found, and occasionally a polymorph contains acid-fast bacilli within its cytoplasm, but the predominant cells are the large mononuclear phagocytes containing numerous acid-fast bacilli.

(iii) *Pancreas, splenic mesentery and omentum.* These are sites of a minor degree of extension from the peritoneal surface to the sub-peritoneal tissues. The lesions here are always minute and are

A NEW MYCOBACTERIAL INFECTION IN MAN

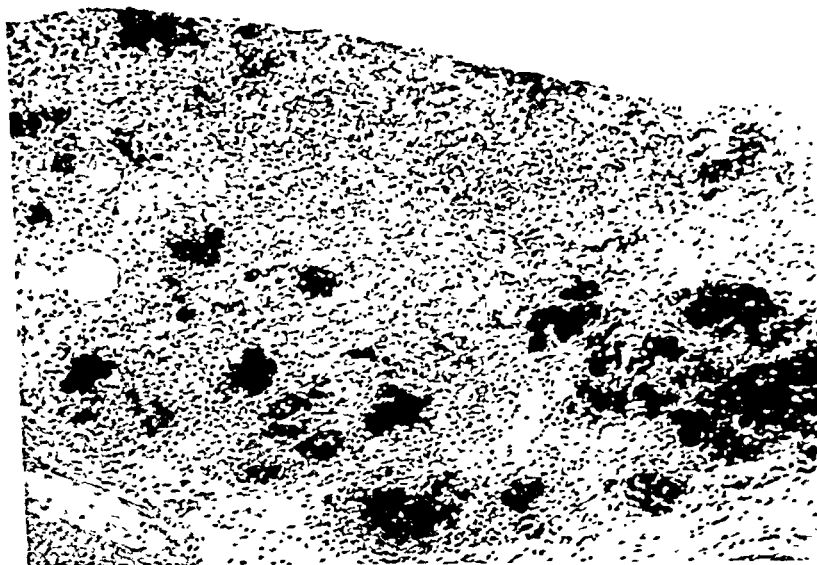


FIG. 12.—Rat inoculated intraperitoneally. Massive accumulations of acid-fast bacilli in lesion of epididymis. Ziehl-Neelsen and hæmatoxylin.  $\times 125$ .



FIG. 13.—Rat inoculated intraperitoneally. Subcutaneous tissue of scrotal wall showing macrophages loaded with acid-fast bacilli. Ziehl-Neelsen and hæmatoxylin.  $\times 150$ .



A NEW MYCOBACTERIAL INFECTION IN MAN

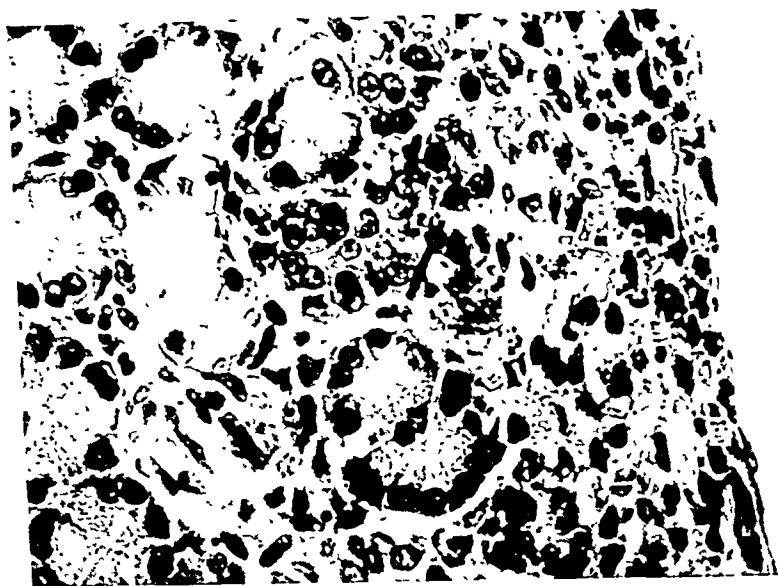


FIG. 14.—Rat inoculated intraperitoneally. Focal lesion of peritoneal surface involving some superficial pancreatic tissue. Hæmatoxylin and eosin.  $\times 450$ .

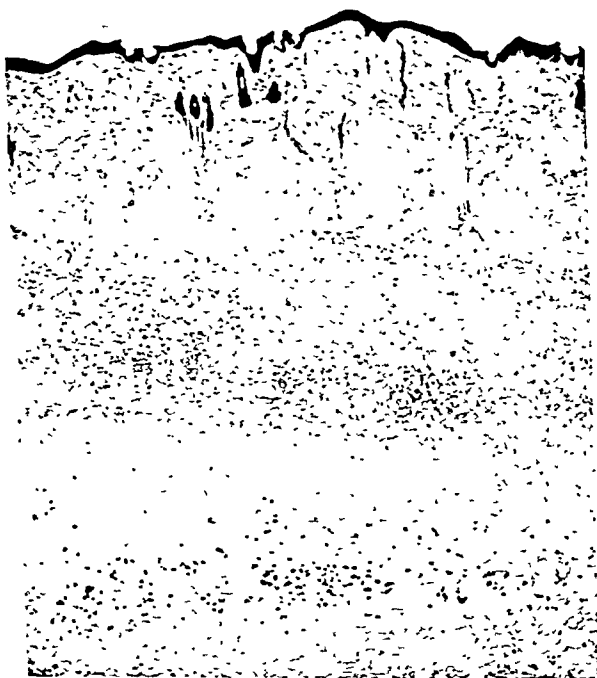


FIG. 15.—Rat inoculated intraperitoneally. Subcutaneous tissue adjacent to an ulcer on the tail, showing inflammatory reaction and general oedema. Dark masses of acid-fast bacilli can be seen in the lower part of the picture. Ziehl-Neelsen and hæmatoxylin.  $\times 33$ .



A NEW MYCOBACTERIAL INFECTION IN MAN

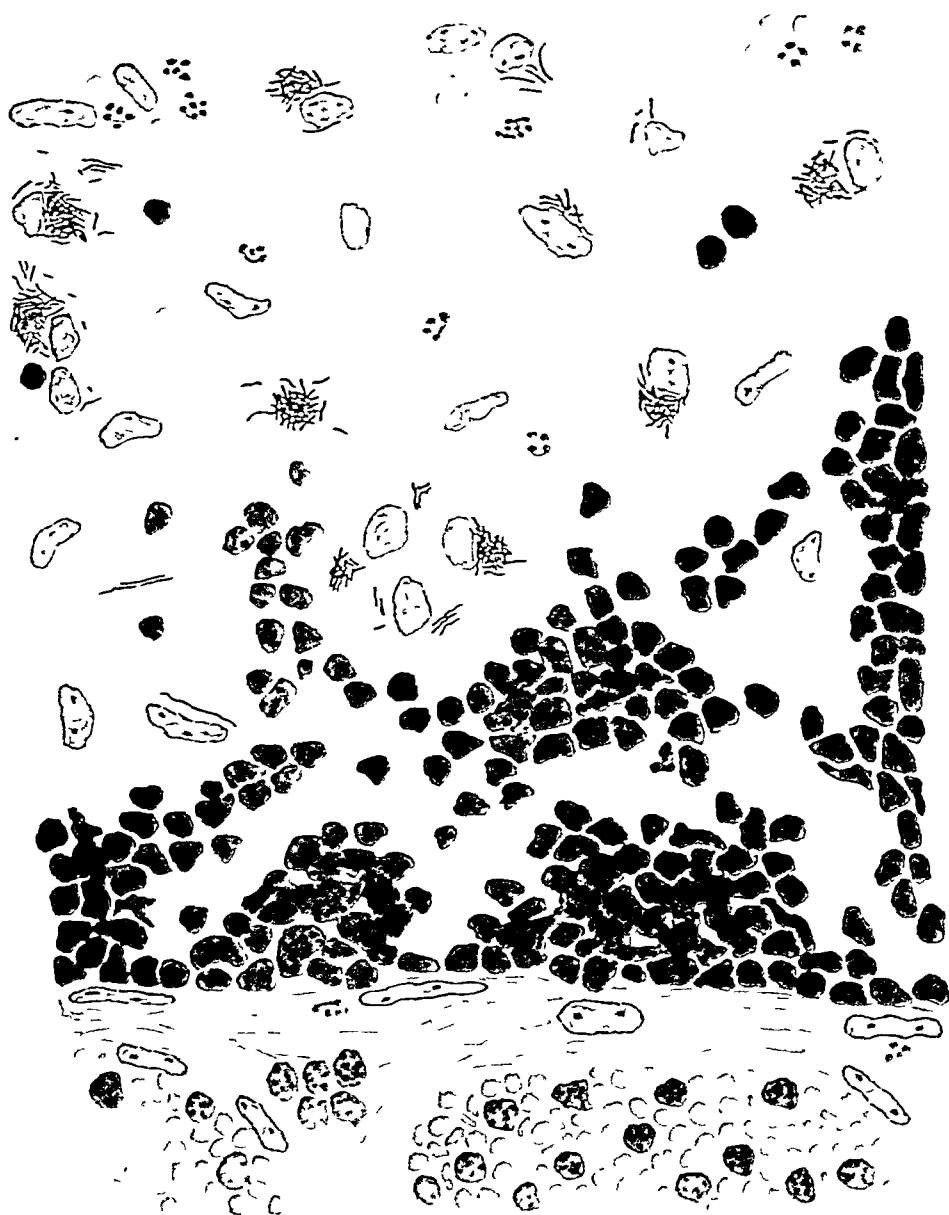


FIG. 16.—Section of cellular membrane covering the spleen surface in a rat inoculated intraperitoneally. Ziehl-Neelsen and hæmatoxylin.  $\times 450$ .





A NEW MYCOBACTERIAL INFECTION IN MAN

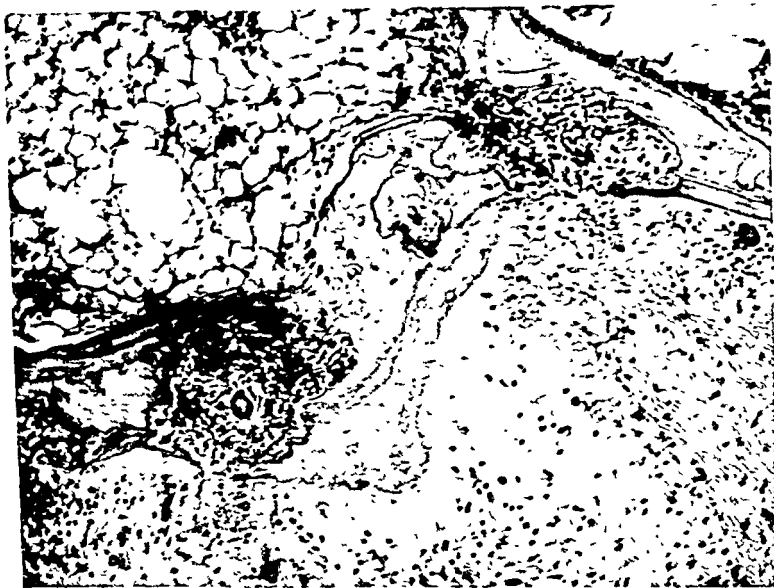


FIG. 17.—Rat inoculated intraperitoneally. Surface of vertebral body adjacent to region of cutaneous ulceration, showing extension to bone. Hæmatoxylin and eosin.  $\times 125$ .



FIG. 18.—As in fig. 17, showing extension of granulation tissue into the bone marrow. Hæmatoxylin and eosin.  $\times 125$ .



found only in occasional animals, contrasting with the gross and constant scrotal lesions in the same animals. Involvement of the pancreas takes the form of patchy destruction of the superficial zone of pancreatic tissue (fig. 14). The acini immediately beneath the peritoneum are destroyed, and their necrotic remains are found scattered between areas of extravasated erythrocytes, nuclear debris and bulky macrophages, some of which contain phagocytosed acid-fast bacilli. In the omentum and splenic mesentery the earliest lesions are seen as small groups of bulky macrophages with many included acid-fast bacilli, surrounded by collections of lymphocytes and unaccompanied by necrosis. Larger lesions are rarely found here, but when present they consist of larger aggregates of bacilli-containing macrophages, with occasional extra-cellular clusters of acid-fast bacilli adjacent to which the connective tissue and fat are disorganised and structureless.

(iv) *Peritoneal lesions in female rats.* It has been described how, in female rats inoculated intraperitoneally, remote lesions occur without the development of either ascites or gross peritoneal disease. No generalised peritoneal deposits are found in these animals, and microscopic examination of the subperitoneal tissues reveals no lesions like those found in males. Indeed the only abnormal findings in this situation were in two rats killed 4 and 9 months respectively after inoculation, in which prolonged search of sections of the various fatty tissues of the abdomen disclosed occasional minute areas of granular change in the fat surrounding the uterine horns and ovaries. In these areas cell outlines are obliterated and granular material replaces the cells. In one rat this material contains scattered acid-fast bacilli, and while no marked inflammatory response is present, an occasional macrophage contains acid-fast bacilli. In the second rat no recognisable acid-fast bacilli can be seen in the same abnormal tissue, but a few areas of granular acid-fast material are found.

#### *Remote cutaneous lesions*

The development of these lesions is a striking though only an occasional feature of the later stages of the experimental disease in rats of both sexes. An animal inoculated intracerebrally developed precisely similar lesions, and the following description applies both to it and to the others of the larger group.

The tail or one of the limbs of an animal may become markedly swollen, often the hair falls out, and the oedema may be followed by ulceration of the skin. In some cases extensive destruction of all the tissues of the part follows, and in one animal the tail and the distal parts of the limbs dropped off (fig. 3). In the early stages the lesions are restricted to the subcutis, and sections of the waterlogged tissues show numerous inflammatory cells but only a few acid-fast bacilli. In ulcerated lesions acid-fast bacilli are usually present in

much greater numbers and are arranged in characteristic extra-cellular masses surrounded by zones of necrotic tissue. On naked-eye examination of such a region, necrosis can be observed to extend as an opaque grey zone in the subcutaneous tissue, as far as 2 cm. beyond the area of ulceration. It is this peripheral zone that is shown in fig. 15.

Thrombosis and ultimate obliteration of blood-vessels is observed and subcutaneous nerves passing through the lesions are destroyed by the necrotic process. In none of the lesions studied, however, could any extension of the inflammatory change along the tissues of involved nerves be found. Muscles and connective tissues are destroyed in the extending lesions, but the periosteum of bones halts the process, at least for a time, and at the surface of a bone it is common to see extension of the lesion arrested over a wide area. The skeletal tissues are, however, occasionally encroached on (figs. 17 and 18), and this occurs most commonly in the region of the spongy bone adjacent to the ununited epiphyses of the long bones of the limbs. The inflammatory tissue extends along the Haversian canals, and the marrow of the involved bones becomes replaced by masses of bulky mononuclear cells containing acid-fast bacilli and eventually by necrotic œdematous tissue containing bulky bacillary aggregates. On the bone surface, in the Haversian canals and in the more central parts of the bones the inflammatory process causes local death of osseous tissue, and necrotic trabeculae devoid of bone cells are found in these situations. Occasionally the involvement of the nutrient vessels by the necrotising inflammatory process leads to necrosis of the entire shaft. In areas adjacent to necrotic bone and in regions where inflammatory tissue is near to living bone, some osteoclastic activity and bone removal is seen. In none of the lesions, however, has any development of new bone been recognised. The epiphyseal cartilages of long bones sometimes remain unaffected, even when the adjacent bone is widely destroyed.

#### *Other disseminated lesions*

The preparation of routine sections of apparently uninvolved organs resulted in the occasional discovery of microscopic lesions in situations other than those already described. These were found in the liver, spleen and peribronchial tissues of the lung (on one occasion each), and took the form of collections of acid-fast bacilli in one or two cells of macrophage type, without any other evidence of tissue reaction. There was nothing to suggest that any of these lesions were progressive.

#### *Lesions following the subcutaneous injection of bacilli*

Sections from several animals which had been injected subcutaneously with material rich in acid-fast bacilli showed local extra-cellular aggregations of bacilli associated with tissue necrosis

and the accumulation of inflammatory cells. Once again these included numerous macrophages, some of which contained acid-fast bacilli. In these lesions, however, bacilli were not so numerous as in the more progressive cutaneous lesions. Indeed, from the histological standpoint these lesions were comparable with those described by Griffith (1911) as the result of the subcutaneous injection of rats with tubercle bacilli.

### Discussion

The outstanding feature of the disease after intraperitoneal inoculation in male rats is the establishment of gross lesions on the peritoneal surface of the scrotum and its contents. The massive peritoneal effusion which develops apparently has its origin in these lesions, which in addition appear to maintain a reservoir of acid-fast bacilli from which colonisation of other parts of the peritoneal surface is effected. The initial localisation in the epididymis and scrotum is surprisingly comparable with the results, described by Thomas (1936), of introducing barium sulphate into the peritoneal cavity of the male rat. Indeed not only were lesions produced in a similar situation by this means, but the cellular reaction to the barium sulphate also consisted of a lymphocyte and macrophage response followed by the development of granulation tissue.

After intraperitoneal inoculation, general dissemination of acid-fast bacilli takes place, but the findings in female rats indicate that this can also occur in the absence of gross peritoneal lesions. The establishment of these lesions, however, as a result of the favourable properties of the scrotal tissues, is responsible for the difference in the course taken by the disease in male and female rats.

When the histological features of the lesions are compared with those of other mycobacterial diseases it must be remembered that the tissues of rats and mice react to a variety of organisms of this group in ways that have many features in common. Thus the accounts given by Griffith (1907), Gloyne and Page (1923), Lange and Simmonds (1923-24), Smith and Hendrick (1925-26), Steinbach (1932), and Hehre and Freund (1939), of the histology of experimental tuberculosis in rats, by Gunn *et al.* (1933-34), Pagel (1940), and Glover (1944) of experimental tuberculosis in mice, by Oliver (1926), Lowe (1934-35, 1937), and Pinkerton and Sellards (1938) of spontaneous and experimental rat leprosy, and by Krakower and González (1937, 1940) of mouse leprosy, show that in all these the macrophage is the most common reacting cell; that its cytoplasm not infrequently assumes a vacuolated or "foamy" appearance; and that the giant-cell systems so characteristic of human tuberculosis are in most cases absent. It is in the light of these distinctive features of mycobacterial infection in rats and mice that the histological changes now described must be judged. It must therefore be concluded that although the histological changes in the present lesions are not exactly duplicated in other mycobacterial

diseases of the rat, it is not so much in the type of cellular response that this particular inflammatory process is distinctive, as in its more general characteristics. Among these the most remarkable are the specificity of site of the lesions and their association with great exudation of fluid.

#### IV. CULTIVATION OF THE NEW MYCOBACTERIUM

GLEN BUCKLE and JEAN C. TOLHURST

Efforts to cultivate the acid-fast bacillus described in sections I and II from the patients S.F. and R.T. and from peritoneal fluid of rats infected from these patients on media suitable for tubercle bacilli having failed, it was assumed that the organism needed some special factor for growth. A variety of media was used in different experiments, including some consisting of one of the conventional bases with additions such as vitamins, sterile human tissues, heated tissue from the ulcer of the patient R.T., *i.e.* a suspension of the organism itself, and so forth. Some of these were incubated at 37° C. in an atmosphere containing carbon dioxide, others in air at room temperature, 37° and 43° C. Culture in the yolk sac of the developing chick embryo at 37° C. was also attempted. All these experiments failed.

Eventually growths were obtained from rat peritoneal fluid on a yolk agar medium. The results were irregular and, although several cultures were obtained from fluids containing both the S.F. and R.T. strains, a long series of failures followed and subcultures were grown with difficulty. As there was no doubt of the authenticity of the cultures, it appeared that the organism had been grown on a medium which did not seem liable to variation from batch to batch, as judged by the simplicity of its preparation and the results obtained on it with tubercle bacilli. It followed that the absence of growth must be due to a failure to meet some requirement other than nutritive.

At this point, the fact that the lesions in man were on the skin of the extremities and those in rats in the scrotum or on the tail and limbs suggested to us that the organism might require a temperature below 37° C. With this came the realisation that the series of successful cultures had been incubated in an apparatus with an unsatisfactory heat circulation and that the return to our usual incubators had coincided with the cessation of positive results. An incubator was therefore adjusted to 33° C. and cultures made from a number of stored fluids; growth was obtained from several. Though growth is at best slower and scantier than that of human tubercle bacilli, no further difficulties have been encountered and the organism was successfully cultivated directly from the ulcer of patient K.R. (case 6).

Our earliest observations being in some details invalidated by doubt as to the exact temperature of incubation, cultures were set up with each of the following :—(a) Suspension of pus and tissue in saline from the patient K.R.

(b) Citrated peritoneal fluid from rat S.F. 51. (c) Citrated peritoneal fluid from rat R.T. 139. (d) Suspension of second generation culture from the patient K.R. (e) Suspension of fourth generation culture from rat S.F. 20. (f) Suspension of sixth generation culture from rat R.T. 62.

The inoculum was one drop from a Pasteur pipette. The number of organisms in the first three inocula was not determined, but fig. 8 indicates the number of colonies obtained on suitable media from fluid (a), which had the fewest viable organisms. The inocula for the secondary cultures were saline suspensions (the largest particles being just visible on shaking) of an opacity equal to our vaccine standard— $2000 \times 10^6$  organisms per ml. In addition, cultures of human and bovine tubercle bacilli from sputum and pus were made on the same media.

All solid media were corked and incubated in a sloping position. The temperatures of incubation mentioned are the means of the temperatures recorded by a maximum and minimum thermometer incubated with the cultures. The general description is of growth at 30 or 33° C.

### *Cultural characteristics*

*Temperature.* Growth was satisfactory at 30 and 33° C., poor at 25 and 37° C. and absent at room temperature (17–23° C.) and at 41° C. On suitable media primary growth may be visible in the fourth week at 30 or 33° C., but in experiments at observed temperatures it appeared in only one of 213 tubes incubated at 37° C. (a single colony, visible after 12 weeks) and in two of 36 tubes incubated at 25° C. for 21 weeks. Secondary cultures, visible in the second week at 30 and 33° C., took 3–4 weeks to appear at 37° and 4–6 weeks at 25° C.

*Yolk agar.* This medium consisted of one part of egg yolk to which was added aseptically three parts of sterile 1·25 per cent. agar dissolved in saline and 1 per cent. of a 2 per cent. solution of malachite green. Primary growth usually appeared in from 22 to 25 days but has taken as long as eight weeks. Colonies when first seen were pin-point, colourless, transparent domes with a smooth surface and entire edge. After nine weeks the colonies were 2–3 mm. in diameter, round, smooth, low convex, opaque and whitish or pale cream in colour; the green dye was sometimes destroyed where growth was heavy. Subcultures were sometimes visible in 11 days. The appearance of the growth was the same. Macroscopically homogeneous suspensions could be made with difficulty from young but not from mature colonies.

*Petragnani's medium.* Growth first appeared in about 30 days, when the tiny colonies were transparent and colourless, dome-shaped and with a smooth surface; later the surface became dull, the outline less regular and the colonies were opaque and cream or pale lemon in colour. When fully grown, colonies were usually low convex or umbonate, but might be flat, 2 mm. in diameter, with a dull rough surface and irregular outline (fig. 8); the colour was a lemon or mustard yellow. Growth appeared in the second week in subcultures and was similar.

*Nutrient agar.* No primary growth was obtained on 2 per cent.



or 1 per cent. nutrient agar. Subcultures usually failed to grow, but on two occasions extremely scanty growth occurred, first visible in nine weeks.

*Nutrient agar plus 5 per cent. glycerine.* No primary growth was obtained. Subcultures usually failed but occasionally scanty growth became visible in the fifth week.

*Blood agar.* On media containing nutrient broth plus 1 per cent. and 10 per cent. horse blood scanty growth appeared in four or five weeks. The colonies, though smaller and fewer, resembled those on yolk agar. When the medium contained 2 per cent. agar, growth was delayed for six or eight weeks and the tiny colonies were flat, rough and irregular. Subcultures grew in four or five weeks.

*Dorset's egg medium.* A few colonies appeared in eight weeks; subcultures grew in four weeks. The colonies resembled those on Petragnani's medium.

*Loeffler's serum.* Scanty growth appeared in eight weeks; the colonies resembled those on Petragnani's medium but were not pigmented. Subcultures grew in five weeks.

*Glycerine potato.* No growth was observed with primary or secondary cultures after 10 weeks' incubation.

*Broth.* No growth was obtained in nutrient broth; subcultures gave scanty growth in tryptic broth. Only when colony fragments were used as inoculum did growth occur in glycerine broth: these increased in size, forming irregular white balls. A colony floated on the surface attached to a fragment of paraffin increased in size but no pellicle was formed. However, if growth as a pellicle can be initiated, subcultures may differ from those described. Growth in serum nutrient or serum tryptic broth occurred as a flaky deposit.

*The effect of glycerine.* Low concentrations of glycerine enhanced growth. The effect was shown, mainly in the later stages, by the continued increase of colony size when colonies on non-glycerinated media had ceased to enlarge, not by the production of more numerous colonies.

Primary inocula failed to grow on Petragnani's medium containing 10 per cent. glycerine. The colonies on this medium containing 5 per cent. glycerine were larger but fewer than those on the glycerine-free medium, while 1 per cent. glycerine induced a definite increase in the bulk of the growth without diminishing the number of colonies. Subcultures sometimes grew in the presence of 10 per cent. glycerine; 1 per cent. and 5 per cent. glycerine produced equally good results, better than those obtained on the medium without glycerine. On yolk agar, primary growth was often prevented by 10 per cent. glycerine; 1 per cent. and 5 per cent. glycerine had little effect on the bulk of the growth, the increase in colony size being offset by the smaller number appearing. Secondary growth was enhanced by 1 per cent. and 5 per cent. glycerine; some inhibition was often noticed with 10 per cent. glycerine. The colonies on Petragnani's medium containing glycerine were more deeply pigmented than those on non-glycerinated media.

*Pigment.* The colour of the mature colonies on Petragnani was usually recorded as pale lemon or lemon, occasionally as resembling

that of dry mustard, once as light tan. The shade varied, but it was always a greenish or brownish yellow, never a golden or orange yellow. On yolk agar there was little or no pigment production—heavy growth sometimes appeared creamy but this might be due to the yellow colour of the medium showing through, the whitish colour being due to opacity rather than a pigment.

### *Pathogenicity for rats*

Pairs of male rats were inoculated intraperitoneally with each of the three strains (0.5 ml. of suspensions *d*, *e* and *f*). The animals were killed when ascites developed 3.5 months later and all three strains were recovered in culture.

In earlier experiments with the S.F. and R.T. strains, peritoneal fluid from rats infected by the intraperitoneal injection of cultures was injected into other animals and fluids from these into a third group. The organisms were not recovered in culture from the first group because an incubator temperature of 37° C. was used, but cultures were obtained from the later groups (*e.g.* fluid *c* from the third animal in such a series). Most of the rats injected died or were killed when ascites was present. In some the complete course of the disease was reproduced.

### *Resistance*

A suspension in saline of culture R.T. 62 was killed by heating to 60° C. for 30 minutes. Growth was obtained from rat peritoneal fluids stored in the refrigerator for periods up to nine months and from the saline suspension of pus from patient K.R. similarly stored for three months. The organism withstands treatment with sulphuric acid.

### *Microscopic appearance*

Since the colonies were difficult to emulsify, low-power views of smears of cultures showed fragments, many of which could be seen to be composed of branching undulating strands resembling the micro-colonies of tubercle bacilli shown by Pryce (1941). In these, the organisms lay more or less parallel. Smaller clumps and groups of bacilli in palisade formation were also seen, but a field such as fig. 11, chosen to show the individual bacteria, was uncommon. In culture, bacillary length was less variable than in the tissues; most organisms were between 1.5 and 3  $\mu$  long and about 0.2  $\mu$  broad. The sides were parallel, the ends rounded and most of the organisms were slightly curved. Ten-day-old subcultures stained for 10 minutes with hot carbol-fuchsin and treated with 3 per cent. hydrochloric acid in 70 per cent. alcohol for three minutes were deeply stained. A few organisms were decolourised in ten minutes but the majority resisted

treatment for at least an hour. The morphology did not appear to vary significantly on the different media used, with the exception of yolk agar containing 10 per cent. glycerine. On this medium swollen elongated forms, some resembling in shape the involution forms of *Corynebacterium diphtheriae*, were seen.

### Discussion

Since only three strains are discussed and the oldest of these is a sixth generation culture, the final description of the organism may differ in detail, but sufficient information is available to enable it to be cultivated and recognised.

The organism shows some notable differences from other mycobacteria. In its lack of resistance to heat and its inability to grow at 47° and 37° C. or at room temperature it differs from many of the saprophytes (Gordon and Hagan, 1938). The only reference we can find to a mycobacterium having a similarly restricted range of temperature for growth is that of Duval (1910); but this is a doubtful instance, for Duval and his collaborators (Duval and Gurd, 1911; Duval and Wellman, 1912; Duval and Holt, 1933-34 *a* and *b*) later used 37° C. for the incubation of primary cultures.

In its nutritive requirements our organism resembles other mycobacterial pathogens. The growth on agar is no more than may be obtained with tubercle bacilli. The requirements for growth probably differ from those of human tubercle bacilli; for although glycerine may enhance growth on other media, its addition does not supply the factors missing from nutrient agar. Blood or a small quantity of egg yolk is necessary for the appearance of more than an occasional colony, and large colonies are obtained only on Petragnani's, yolk-agar or similar medium.

On injection into rats cultures produce a progressive, transferable infection with distinctive characteristics. The temperature requirements offer an interesting parallel with the sites of the lesions. The relative insusceptibility of female rats, the sequence of events in male rats and the resistance of the viscera in both sexes, especially to the heavy secondary inoculation which must occur in a male when ascites develops, are all suggestive of the influence of the temperature of the site on the establishment of a lesion. The lesions in man have also occurred in situations where the temperature is usually below 37° C.

These studies will be continued and serological investigations have been commenced. We do not, at present, propose a name for the organism.

### V. SUMMARY

In a series of 6 cases of ulceration of the skin occurring in a rural area in Australia, a mycobacterium hitherto unrecorded and pathogenic to man was found in the lesions.

The lesions are described, the relevant clinical data are recorded, and an account is given of the laboratory investigations which have been carried out.

In the three cases in which detailed laboratory studies were made, material from the ulcers infected rats and mice but not guinea-pigs. In rats characteristic lesions were produced, including ascites, cutaneous oedema and ulceration, and the disease was transferable from rat to rat indefinitely.

The organism, a strongly acid-fast mycobacterium, has been cultivated and its characteristics are described. It requires a temperature below 37° and above 25° C. for its growth (optimum about 33° C.), and in its nutritive requirements it is fastidious to a degree comparable with the tubercle bacillus.

The relationship of this disease to other mycobacterial infections is discussed, and it is concluded that the causal organism is distinct from any of the hitherto described mycobacteria.

Comment is made on certain implications of finding a new mycobacterium pathogenic for man.

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## THE ANTIBODY RESPONSE IN CASES OF RADIATION LYMPHOPENIA, AND IN THE RETICULOSES

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### Antibody response in radiation lymphopenia

For many years evidence has been accumulating that the lymphatic system plays an important part in immunity reactions. Hektoen (1915) showed that in rats exposed to X-radiation a decrease in hæmolysin production occurred and that this was associated with a corresponding reduction in the number of circulating lymphocytes. He concluded that antibodies are formed in the spleen, lymphatic tissue and bone marrow, since these are the structures most affected by radiation. Rabbits were exposed to X-radiation by Murphy and Sturm (1925) until they showed a pronounced lymphopenia. In these animals a deficiency in the production of precipitins, bacterial agglutinins and protective bodies was recorded. McMaster and Hudack (1935) showed, in mice, that antibodies first appeared within the regional lymph nodes draining the site of injection of the antigen, and that if two different antigens were injected, one into each ear, the corresponding antibody first appeared in the lymph glands of the same side. Ehrich and Harris (1942) demonstrated that the tissue response accompanying the formation of antibodies in the lymph node was chiefly a diffuse lymphocytic hyperplasia, followed by the appearance of large reactive germinal centres and a sharp rise in the output of lymphocytes into the efferent lymph. Kass (1945) demonstrated the occurrence of gamma globulins in lymphocytes and suggested that this fact, taken in conjunction with the evidence of the previous authors and the failure of lymphocytes to absorb or adsorb antibody *in vitro*, shows that the specific alteration of gamma globulin to cause the molecule to become reactive towards a given antigen occurs within the lymphocyte.

There is therefore evidence to suggest that a possible correlation may exist between the amount of active lymphatic tissue in the body and the antibody response to a given antigen. Patients receiving large doses of X-radiation for the treatment of neoplastic disease develop a pronounced lymphopenia. This persists for several weeks after the cessation of treatment and has been a matter of great interest to both radiologists and hæmatologists for many years. The exact significance of this lymphopenia as an indicator of the systemic effects of radiation therapy is largely speculative, but nevertheless it has for some time been used as a guide to the amount of radiation to which a patient can be exposed without permanent or lethal effects.

The exact fate of the lymphocytes that disappear from the circulation during radiation is unknown. Many appear to be damaged or destroyed in the circulation, but it is possible that, after the destruction of the existing hæmic lymphocytes during the early phases of radiation, further lymphocytes do not reach the blood stream. Indeed they may never be formed, or, if formed, only in reduced numbers, as the lymphogenic centres become themselves affected by radiation (Goodfellow, 1936). If then, as recent evidence suggests, the seat of antibody elaboration is the lymph node, the measure of failure of antibody production would be related to the amount of damage sustained by the lymphatic system from radiation.

With this in view it was considered worth while to investigate the antibody response in 30 patients receiving large doses of X-radiation and to correlate the results with the severity of the induced lymphopenia.

#### METHOD

Only patients giving a negative history of enteric fever who had received no inoculation of any sort were chosen. The test series was comprised almost entirely of cases of carcinoma of the ovary and seminoma of the testis undergoing regional X-ray therapy. The control group consisted of 17 uninoculated healthy adults of both sexes and of patients with malignant and other diseases, not undergoing radiation treatment, who showed no evidence of lymphopenia.

A standard agglutinable suspension of *Bact. paratyphosum* A(H) (Standards Laboratory, Oxford) was chosen as the test antigen, chiefly because paratyphoid A is a rare form of enteric disease in this country, and also because higher titres could be obtained by using the flagellar antigen. In each instance 1.0 ml. of antigen was injected intramuscularly into the arm. A preliminary agglutination reaction using a macroscopic volumetric technique was carried out on blood specimens obtained immediately before the initial injection. It was invariably negative. Further agglutination reactions were carried out on specimens of sera collected at intervals of 7, 14 and 21 days after the initial injection. The results were recorded as titres expressed in dilutions of serum.

It was not always possible to obtain all four samples of blood, as patients were either discharged from hospital before the completion of the experiment or failed to appear at the out-patient clinic.

Blood counts were carried out at the time of injection of the antigen and subsequently at 7-day intervals. The experiments were usually commenced when the lymphocyte level had fallen to approximately 500 cells or less per c.mm. This generally corresponded to the time when radiation was about to be discontinued, either because the number of exposures had been completed or the lymphocyte level had fallen too low for further therapy with safety.

#### RESULTS

After seven days the most frequent result recorded in the test series was that of no agglutination (0); this occurred 12 times out of 30 determinations. Ten showed titres of from 1:4 to 1:8 only, 7 showed titres ranging from 1:16 to 1:64, and one only a higher titre (1:128). The control series of 17 adults gave titres ranging from 1:8 to 1:16,384 seven days after injection of the same quantity of antigen. The most frequent titres recorded in the control group

TABLE I

No.	Disease etc.	W.B.C.	Polys.	Lymphs.	Titres in dilutions of sera after (days)		
					7	14	21
Lymphopenic group							
1	Ca. of ovary	4000	2800	370	0	40	16
2	" "	2900	2800	175	0	256	...
3	" "	3500	3100	72	0	2048	...
4	" "	3900	3200	211	0	8192	...
5	" "	3500	2600	480	16	2048	1024
6	Seminoma	4000	2800	235	0	4096	16384
7	Ca. of ovary	2900	2300	235	0	80	64
8	Seminoma	3400	2610	333	128	8192	16384
9	"	2900	2100	246	4	4	0
10	"	5000	3800	693	8	8192	...
11	Ca. of ovary	3800	2800	400	8	2048	1024
12	" "	4300	3200	180	4	2048	768
13	" "	5000	4200	280	64	512	...
14	" "	3400	3000	153	4	192	512
15	" "	4300	3450	420	0	2048	2048
16	" "	2900	2100	240	4	128	...
17	" "	2200	1700	200	8	256	4096
18	" "	3800	2900	240	16	...	...
19	" "	4200	3000	310	0	8192	...
20	" "	3800	3300	240	8	8192	...
21	" "	4900	4000	290	32	8192	1024
22	" "	2900	2170	340	0	192	...
23	Seminoma	2600	2000	200	64	64	64
24	Ca. of ovary	5100	4300	230	0	128	128
25	" "	3100	2500	230	8	128	128
26	Ca. of bladder	7900	6500	225	16	348	512
27	Ca. of ovary	2800	1700	590	64	128	...
28	Myeloma	2700	2100	160	0	256	512
29	Sarcoma	5800	4570	128	0	256	512
30	Ca. of ovary	4100	3460	200	8	256	...
Control group							
1	Ca. of bladder	10000	7800	2180	128	16384	32768
2	Ca. of mouth	6700	4800	1500	8	16384	16384
3	" "	14900	11500	2280	12188	16384	32768
4	Ca. of bladder	12400	9860	2110	2048	1024	512
5	Ca. of mouth	9700	7810	1160	2048	24576	32768
6	Lab. Asst.	6000	3400	2160	256	1024	2048
7	Doctor	6500	3370	2280	1024	32768	4096
8	Student	6500	3470	2180	64	8192	16384
9	Ca. of bladder	10000	6000	3000	8192	65536	32768
10	Student	8000	5600	2000	4096	8192	...
11	"	8200	5660	2050	4096	8192	...
12	Lab. Asst.	6200	4030	1674	2048	16384	...
13	Student	7400	4810	2000	8192	16384	...
14	"	8600	5300	2600	4096	8192	...
15	"	6500	3900	1950	16384	65536	...
16	"	7200	5040	1800	2048	4096	...
17	Banti's syndrome	4600	2600	1426	4096	8192	...

The figures for the leucocytes are the average of four counts at 7-day intervals, commencing at the time of inoculation.



were 1:2048 and 1:4096. Only 2 individuals gave titres of below 1:128, whereas 13 showed titres above 1:512.

The differences in titres between the two groups 14 days after injection were still apparent. The most frequent titres in the test series were from 1:128 to 1:512, which were recorded in 13 out of the 29 cases examined. Four patients gave values ranging from 1:4 to 1:64, 6 from 1:1024 to 1:4096, and 6 showed agglutination at 1:8192, which was the highest titre recorded for this group. The corresponding values in the control series ranged from 1:1024 to 1:65,536, the most frequent values (14 out of 17) ranging from 1:8192 to 1:65,536.

The number of determinations carried out in both test and control groups for the 21-day period were much fewer in number. Out of the 18 results for the test series, 11 ranged from "no agglutination" (0) to 1:512, and 7 from 1:1024 to 1:16,384. The most frequent result was 1:512. Of the 9 tests carried out in the control group 6 gave values ranging from 1:16,384 to 1:32,768; the other 3 gave readings of 1:512 to 1:4096.

The detailed results are shown in table I. In each case only the average figures for the four leucocyte counts are given, as the variations recorded in the consecutive counts at 7-day intervals were not substantial. In the lymphopenic group there was a tendency for the leucocytes to diminish still further during the course of the experiment. The average total leucocyte counts in the test group ranged from 2200 to 7900 (polymorphs 1700-6500, lymphocytes 72-693, monocytes 140-550) per c.mm. The corresponding values for the control group were:—total leucocytes 4600-14,900, polymorphs 2600-11,500, lymphocytes 1160-3000, monocytes 130-740 per c.mm.

### DISCUSSION

The results suggest that there is a delay in the production of antibody and a diminution in the amount produced in the test series as compared with the controls, and that this is associated with a reduced number of circulating lymphocytes. The differences in titre are especially marked when results obtained 7 days after injection of antigen are compared. It can of course be pointed out that during this period the polymorphonuclears and the monocytes were also reduced in number, and further that the inhibition of antibody production could be explained by the partial blockage of the reticulo-endothelial system by the cellular debris of organs and remains of broken down lymphocytes caused by radiation. That this could not be the correct explanation is suggested by the experiments of Murphy and Sturm, who exposed rabbits to dry heat before and after the injection of an antigen. These animals developed a marked lymphocytosis and produced antibodies in far higher titre than rabbits not so exposed. As there is no evidence that dry heat has any effect

on macro- and microphages the response of these animals to dry heat cannot be explained by hyperactivity of the reticulo-endothelial system. The cellular response within lymph nodes during antibody production is lymphocytic (Ehrich and Harris), and this is followed by an outpouring of antibody into the efferent lymph (Harris *et al.*, 1945). The same workers have also shown that granulocytes and macrophages do not contain antibody, even during the period of maximum agglutinin production, and that they do not synthesise bacterial agglutinin against typhoid and dysentery bacilli (Ehrich *et al.*, 1946).

The more probable explanation is that suggested by Bunting (1938), namely that the micro- and macrophages play no part in the elaboration of antibodies, but by breaking down antigens into simpler substances prepare them for utilisation by the lymphocytes. X-radiation, by its effect on the circulating lymphocytes and on those in the lymph nodes, would cause an inhibition of this function of the lymphocyte. The delay or failure of antibody response would then be proportional to the damage sustained by the lymphatic system as a result of radiation.

#### Antibody production in the reticuloses

Proliferative diseases of the reticulo-endothelial system cause large scale replacement and destruction of the lymph glands. To ascertain the extent to which this process would be associated with failure of antibody production, a group comprising 5 cases of lymphosarcoma, 2 of lympho-follicular reticulosis, 3 of Hodgkin's disease, 7 of chronic myelogenous leukæmia and 5 of chronic lymphogenous leukæmia were subjected to the same test for agglutinin response. Some of these cases were being treated by chemotherapy, some by radiation.

With the exception of the 7 cases of myelogenous leukæmia, which were being treated by urethane, the titres obtained compared very unfavourably with the control series, the majority giving responses considerably lower than those recorded in the lymphopenic group. No significance is attached to the treatment of the myelocytic leukæmias by urethane in accounting for the higher titres obtained in this group. The results are given in table II.

Moreschi (1914) observed that a patient suffering from lymphatic leukæmia who contracted typhoid fever failed to develop antibodies against *Bact. typhosum*. He found from the literature that about 6.5 per cent. of patients suffering from typhoid failed to show specific agglutinins, and during the course of two years he inoculated with *Bact. typhosum* 2 cases of lymphatic leukæmia and 6 of myelocytic leukæmia to find out whether or not the failure of antibody response in his original case was likely to be due to the leukæmia. Two of these cases had been treated by X-rays and developed agglutinins to 1:20. The others showed no evidence of agglutinin formation. During the same period he observed a patient with lymphosarcoma who had acquired a paratyphoid B infection and who failed to develop agglutinins.

were 1:2048 and 1:4096. Only 2 individuals gave titres of below 1:128, whereas 13 showed titres above 1:512.

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Howell (1920) obtained similar results in one case each of lymphogenous and myelogenous leukaemia, using T.A.B. as the antigen. The loss of ability to form agglutinins was attributed to the excessive proliferation of the haemopoietic system, one of the normal functions

TABLE II

No.	W.B.C.	Polymorphs	Lymphocytes	Disease	Titres after (days)			Treatment
					7	14	21	
1	146,000	94,700	1500	Myel. leuk.	8	9192	16,384	Urethane
2	86,000	38,500	1250	" "	8	384	128	"
3	10,800	9200	117	" "	0	512	1536	"
4	121,000	88,000	1940	" "	16	1024	10,240	"
5	100,500	59,000	3100	" "	1024	10,240	10,240	None
6	309,400	212,500	7050	" "	8	1024	128	Urethane
7	53,000	36,000	1220	" "	4	128	128	X-ray
8	48,000	4800	41,760	Lymph. leuk.	0	0	0	X-ray and urethane
9	48,000	5760	42,240	" "	4	3072	16,384	Urethane
10	74,500	9313	62,200	" "	0	0	0	X-ray
11	100,000	10,000	88,000	" "	0	0	0	Urethane
12	120,000	6000	111,600	" "	0	2	64	"
13	6700	5494	603	Lymphosarcoma, tonsil	64	2048	512	X-ray
14	5400	3868	1134	" "	32	32	0	"
15	4000	3600	80	" "	0	64	...	"
16	23,200	12,800	10,400	Ret. cell sarcoma	0	0	0	Old X-ray
17	7000	3479	3266	Early "Hodgkin"	0	384	256	Urethane
18	9500	8300	712	"	0	512	2048	Nitrogen-mustard
19	3400	2700	294	Late Hodgkin	0	0	0	"
20	5650	4144	700	"	0	0	0	None
21	5200	2990	1320	Lymphofoll. reticulosis	0	80	1024	X-ray
22	4600	2484	1656	Lymphofoll. reticulosis and sarcoma	0	0	0	None

of which was supposed to be that of antibody formation. Unfortunately she used a micro-technique and probably did not allow for the pro-zone phenomenon which in the present series was frequently observed up to dilutions of 1:8 in cases giving positive results.

Bernstein (1934) found that the increase of antibody concentration which normally follows the intravenous injection of horse serum did not occur in 2 cases of leukaemia. Weinstein and Fitz-Hugh (1935) found that the parenteral administration of horse serum did not produce a rise in heterophil antibody titre in 5 cases of lymphatic leukaemia. A similar failure to increase heterophil antibody by the injection of horse serum was found in one case of Hodgkin's disease and in 2 cases of lymphosarcoma. The authors suggest that this indicates a biological relationship of these conditions to lymphatic leukaemia. The parenteral administration of horse serum to 3 cases of myelogenous leukaemia produced a marked rise in heterophil antibody, of the same order as

that produced in healthy adults. This fact Weinstein and Fitz-Hugh take to demonstrate a biological difference between the myelogenous and the lymphatic types of leukaemia.

### DISCUSSION

Lymphadenopathy is usually a late manifestation of myelocytic leukaemia and is generally slight or moderate in degree. Marked enlargement is rare. On the other hand the most striking feature of lymphatic leukaemia is the early enlargement of all lymph glands. Extensive lymph-glandular enlargement is also the chief feature of lymphosarcoma and Hodgkin's disease. From the histological standpoint there is no sharp distinction between lymphocytic leukaemia and lymphosarcoma. In both conditions the normal gland structure disappears and the germ centres are not distinguishable. In Hodgkin's disease the lymphoid tissue is replaced by proliferation of the reticulo-endothelial cells and by fibrous tissue. The lymph glands in myelocytic leukaemia retain their pattern for a longer period: obliteration of the follicles when it occurs is a late event.

It would not be unreasonable to assume that conditions which obliterate the architecture of the lymph gland would also destroy its function. This would account for the marked failure of antibody response in lymphatic leukaemia, Hodgkin's disease and lymphosarcoma, as the cells in the lymph nodes in these conditions are abnormal, and however much they may resemble lymphocytes, they are incapable of antibody synthesis. In myelocytic leukaemia the retention to a greater extent of the morphology and function of the lymph glands would explain the higher titres obtained in this type of reticulosis.

In this small series the titres were lower in the late cases of Hodgkin's disease than in the one early case included. Similarly there were slightly higher titres recorded for the cases of lymphosarcoma involving the tonsillar region than for the more generalised form of the disease. The uncomplicated case of lymphofollicular reticulosis showed some evidence of antibody production, whereas the other, showing sarcomatous changes, gave no agglutinin response. The failure of antibody formation in this group might well be related to the degree and extent of replacement of the normal lymphatic tissue by abnormal cells.

### SUMMARY

The antibody response was tested in a series of 30 patients exhibiting a radiation-induced lymphopenia and in a control group of 17 individuals with a normal blood-lymphocyte level. A suspension of *Bact. paratyphosum* A(H) was used as antigen. A lower level of antibody production was recorded in patients showing a low lymphocyte count. The significance of this finding is related to the immunological function of the lymphocyte.

A series of reticulo-endothelioses comprising cases of myelocytic and lymphatic leukæmia, Hodgkin's disease, lymphosarcoma and lymphofollicular reticulosis was also tested for agglutinin response, which was found to be very poor except in the myelocytic leukæmias. This poor response is attributed to the degree of disorganisation of the lymph glands in these conditions.

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## SHORT ARTICLES

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### LEUCOCYTES AND BACTERIA IN HUMAN SEMEN

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Microscopic examination of human semen always reveals the presence of leucocytes. Their number ranges widely in different specimens and in poor semen they are often numerous enough to indicate that the physiological limit has been surpassed, although the average number of leucocytes in human semen is not stated in the literature.

The purpose of the present work was to determine the average number of leucocytes in samples of human semen which from other criteria were regarded as of normal fertility, to examine if an inverse relation could be disclosed between the number of leucocytes and number of spermatozoa, and to decide if any causal relationship could be established between the bacterial flora of the semen and the number of leucocytes present.

#### MATERIAL AND METHODS

One hundred and twenty-eight samples of semen were examined from different men. Of these, 53 proved to be within the normal range as regards quantity, number of spermatozoa, proportion of motile spermatozoa and differential count. Seventy-five samples were of poor quality or even aspermic (table I). The leucocytes were counted in a Türk counting chamber after careful stirring of the semen and subsequent dilution to 1 : 10.

Bacteriological examination was performed on 50 samples, 18 normal and 32 pathological. Aerobic blood-agar plates were examined after 24 hours at 37° C. and anaerobic cultures after 48 hours. Finally a McLeod-Reyman's plate was examined for gonococci after 24 hours at 37° C. in an atmosphere of 10 per cent. CO<sub>2</sub>.

#### RESULTS

##### *Number of leucocytes in normal semen*

As is evident from table I, the 53 normal specimens with 60 million or more spermatozoa per c.c. showed leucocyte counts that ranged from below 1000 to more than 10,000 per c.mm. The figures include polymorphonuclear and mononuclear types.

The mean value for 53 normal specimens is 3325 leucocytes per c.mm. with a standard deviation of 2925 cells and a standard error of  $\pm 402$  cells. Thirty-seven normal samples (70 per cent.) are dispersed within a range of twice the standard deviation, which indicates a normal distribution.

Little attention seems to have been paid to the number of leucocytes in other secretions, which might be used for comparison with this material. Laquer



(1912) stated that the number of corpuscular elements in the saliva ranged between 350 and 4800 per c.mm., the average of 33 counts being 2000. In the

TABLE I

*Number of leucocytes in relation to number of spermatozoa in 128 samples of human semen (75 pathological and 53 normal)*

No. of spermatozoa (millions per c.c.)	No. of samples	No. of leucocytes per c.mm.							
		500-1000	1000-2000	2000-4000	4000-6000	6000-8000	8000-10,000	10,000-14,000	Mean value
Pathological samples									
Aspermia	23	2	8	6	2	2	1	2	5450
0-1	6	1		2	2	1			4120
1-5	9		1	4	1	2	1		4700
5-10	3				2			1	7466
10-20	8		4	2				2	4500
20-40	7			4		2	1		5150
40-60	19	4	6	6	2	1			2520
75		Mean of 75 pathological samples = 4463							
Normal samples									
60-80	32	6	9	7	6	2	1	1	3280
80-100	18	2	7	5	2	1		1	3170
100+	3	1	1					1	4750
53		Mean of 53 normal samples = 3325							

gastric juice Westermann (1938) counted from 25 to 500 cellular elements per c.mm., 15-25 per cent. being leucocytes, with an increase to 30 per cent. in cases of gastric ulcer.

#### *Number of leucocytes in poor and aspermic semen*

This group, with less than 60 million spermatozoa per c.c., includes 75 samples, of which 23 are aspermic.

The mean figure for leucocytes per c.mm. in the different sub-groups (table I) is a little higher than the mean value of normal semen; but the number of samples in each sub-group is too small to establish any conclusive statistical difference, with the one exception of the aspermic specimens. If this sub-group of 23 specimens with a mean count of  $5450 \pm 766$  leucocytes per c.mm. is compared with the 53 normal specimens—mean count  $3325 \pm 402$  leucocytes per c.mm.—the difference is highly significant ( $t = 2.6864$ ;  $P < 0.01$ ).

If the borderline group with 40-60 million spermatozoa per c.c. is excluded, the mean value of the remaining 56 samples is  $5120 \pm 457$  leucocytes per c.mm., which also represents a statistically significant difference from the normal.

#### *Number of leucocytes in relation to the bacteriological findings*

The origin of bacteria found in semen can never be ascertained with absolute certainty because of contamination from the urinary passages. But if the increased number of leucocytes is due to a bacterial inflammation, a correlation

between the leucocyte count and the bacterial flora would be expected. The low number of leucocytes rules out an acute infection, but a chronic inflammatory process might be regarded as a possible source of these cells.

Table II shows the different bacteria which were found in 18 normal and 32 pathological specimens, as well as the incidence of each species and the

TABLE II

*Number of leucocytes in relation to bacteriological findings in 18 normal and 32 pathological samples of human semen*

Bacteria	Incidence (per cent.)	Number of leucocytes per c.mm.							
		500-1000	1000-2000	2000-4000	4000-6000	6000-8000	8000-10,000	10,000-14,000	Mean value
<i>Staph. albus</i> . .	88	2	10	10	8	5	3	6	5040
<i>Corynebacterium</i> .	76	3	8	9	6	4	4	5	4100
<i>Gaffky</i> . .	20	1	2	5	1	1			3150
<i>Strep. pyogenes</i> .	42			1	1				4100
<i>Neisseriaceæ</i> *	14		3	2				3	5750
<i>Staph. aureus</i> . .	2					1			7200
<i>Strep. faecalis</i> . .	30		5	4	2	3	1		4100
<i>Bact. coli</i> . .	14		3	1	2			1	4200
<i>Hæmophilus</i> . .	4			2					3200
<i>Alcaligenes</i> . .	2				1				4800
Mean of 50 samples = 4470									

\* *N. gonorrhææ* was not found in any of the specimens examined.

corresponding number of leucocytes in the specimen. The mean value of leucocytes in each sub-group does not differ markedly from the mean value of the whole group ( $4470 \pm 473$  cells per c.mm.) and statistically the differences are without significance.

Table III shows the relation between the bacteriological findings and the number of spermatozoa per c.c. Evidently no correlation exists between the number of spermatozoa and the different species of organisms. In table III the proportion of normal and of motile spermatozoa is also given. It should be observed that in the specimens containing *Bacterium coli* the number of motile spermatozoa is notably small, in contrast with the normal number of morphologically normal spermatozoa. Since most of the samples were about one hour old when the examinations started, the poor motility might arise from the production of acid by *Bact. coli*; for these samples showed a lower pH than the others when examined with the potentiometer.

This indicates that comparatively low motility of spermatozoa should be interpreted with due reserve if examination of the semen has been delayed, since contamination with *Bact. coli* may be responsible.

#### SUMMARY

In 53 different samples of human semen of apparently good quality the number of leucocytes ranged between less than 1000 and more than 10,000 per c.mm., the mean value being  $3325 \pm 402$  cells and the standard deviation 2925 cells.

In a total of 75 samples of pathological semen, namely 23 aspermic and 52 poor specimens, the number of leucocytes was increased. The 23 aspermic samples, alone and together with the samples containing less than 40 million spermatozoa per c.c., showed a statistically significant difference from the normal values.

TABLE III

Relation between number of spermatozoa and bacteriological findings in 18 normal and 32 pathological samples of human semen

No of samples	Spermatozoa			No of samples with bacteria									
	No. in millions per c.c.	Normal (per cent )	Motile * (per cent.)	<i>Staph. albus</i>	<i>Corynebacterium</i>	<i>Gaffkya</i>	<i>Strep. pyogenes</i>	<i>Nisseriaceae</i>	<i>Strep. faecalis</i>	<i>Bact. coli</i>	<i>Hemophilus</i>	<i>Staph. aureus</i>	<i>Alcaligenes</i>
10	Aspermia	0	0	10	10	2	1	1	3		1		
1	0.1	60	14	1	1								
3	1.5	18, 29, 40	10, 25, 20	3	2	1		1	1				
3	5.10	08, 52, 02	28, 18, 11	1	2				1	1		1	
3	10.20	50, 37, 01	30, 10, 11	3	3	1	1	1	1				
4	20.10	52, 70, 73 08	15, 30, 15 12	5	1	2			1				
8	40.60	76, 72, 74 60, 61, 63 78, 65	52, 41, 38 15, 30, 10 5, 15	5	3	1		2	2	2	1		
10	†60.80	79, 57, 73 08, 08, 82 83, 78, 75 75	00, 15, 11 28, 24, 18 15, 45, 12 52	11	9	1		2	1	2			1
7	80.100	76, 78, 51 65, 55, 73 76	37, 10, 10 15, 33, 18 38	5	1	2			1	1			
1	100+	81	5						1	1			

\* The heavy type in column 4 indicates specimens yielding *Bact. coli*.

† Specimens having 60 million or more spermatozoa per c.c. are taken as normal.

Bacteriological investigation of 50 different specimens of semen did not disclose any relation between the bacteriological findings and the number of leucocytes. In consequence it may be assumed that the increased number of leucocytes in poor semen depends on non-infective, and probably degenerative, processes.

The specimens containing *Bact. coli* showed low motility, possibly due to acid production.

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# CATALASE PRODUCTION BY GRAM-POSITIVE COCCI: A SIMPLE TEST FOR DIFFERENTIATING ENTEROCOCCI FROM MICROCOCCI

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In the generally accepted classification of the Gram-positive cocci (Coccaceæ) the streptococci are distinguished from the other members of the family by their ability to form chains in culture. The genus *Streptococcus* has now come to include the pneumococcus and the enterococcus, neither of which shows invariably a strong tendency to chain formation. Apart from chaining, it would appear that no important cultural, biochemical or antigenic features have been stressed as being shared by all the members of the genus *Streptococcus*. Sometimes considerable investigation is required before a conclusion can be reached that an organism should be classed as an enterococcus.

During observations on peroxide production by streptococci the use of the catalase test suggested itself as a satisfactory differential character. Apart from the original work of McLeod and Gordon (1923) in which *Staphylococcus* and *Sarcina* are described as catalase-positive and *Streptococcus* and pneumococcus as catalase-negative, no references have been found to a systematic investigation of catalase production by the aerobic Gram-positive cocci. Also none of the standard text-books of bacteriology mention the catalase test in a classification of these organisms.

## Methods

A few c.c. of a fluid culture in broth or peptone water or an agar-slope culture is convenient for the test. One to two c.c. of a 10-volumes hydrogen peroxide solution are added and the evolution of gas is noted. Similarly, a single colony from a Petri plate may be removed by a platinum loop and introduced into a test-tube with 1 c.c. of peroxide solution; in selecting colonies from blood agar care must be taken not to touch the medium. Occasionally the platinum wire may decompose peroxide very slowly, with the evolution of tiny bubbles of gas. This difficulty can be avoided by emulsifying the colony in a drop of peroxide solution on a clean glass slide, when examination by naked eye or with a hand lens will show whether bubbling occurs.

*Genus Streptococcus.* One hundred and sixteen strains have been tested, comprising 54 strains of streptococci (including Lancefield's groups A, B, C, E, G, H, L and N, 23 hæmolytic and 31 non-hæmolytic and *viridans*); 20

strains of pneumococci (including types I, II, III, V, VI, VIII, X, XI, XVI and XXI, and two strains not typed); and 42 strains of enterococci (of which all were resistant to heating at 56° C. for 30 minutes, 40 fermented mannitol (Dible, 1929), 40 produced acetyl methyl carbinol (Barritt, 1936; Lominski, Harper and Isaacs, 1946), 11 were hæmolytic, 12 belonged to Lancefield's group D and one each to group E and group F: one strain of *Streptococcus liquefaciens*, group D, was also included). All were catalase-negative.

*Genus Micrococcus, Staphylococcus and Sarcina.* Of 146 strains tested all produced catalase. They consisted of (a) micrococci, 75 (i.e. organisms which form clusters and do not produce chains, but are distinguished from the potentially pathogenic staphylococci by one or more of the following characters: large size of the individual cocci in culture, failure to develop hæmolsin or coagulase, absence of pigment or production of pigment that tends not to be of the *aureus* colour); (b) pyogenic *Staphylococcus aureus*, 60; and (c) *sarcinæ*, 11.

A more detailed identification of the micrococci and *sarcinæ* was attempted on the basis of the classification given by Bergey *et al.* (1939), when the following varieties were found:—*Staph. epidermidis*, *Micrococcus flavescens*, *halophilus*, *candidus*, *ochraceus*, *cereus*, *conglomeratus*, *cinnebarcus*, *rhodochrous*, *epimethicus*, *nitrificans*, *subcitreus*, *flavus*, *percitreus*, *aurantiacus*, *xenopus*, *subflavescens*, *freudenreichii*, *varians*, *luteus* and *epidermidis*, *Gaffkya tetragena* and *vernetii*, *Sarcina flava*, *subflava*, *lutea* and *urceæ*.

### Summary

One hundred and sixteen strains showing the morphological and cultural attributes of the genus *Streptococcus* are all catalase-negative; in contrast, strains classified as *Micrococcus*, *Staphylococcus* and *Sarcina*, of which 146 were tested, are all catalase-positive. The presence of catalase is of fundamental importance to the metabolism of the bacterial cell and its ability to survive in culture. It seems reasonable, then, to separate the members of the genus *Streptococcus*, which do not produce catalase, from the catalase-positive *micrococci*.

Apart from its interest in classification, the catalase reaction has been found a useful practical test in routine laboratory work. Its great advantage is its extreme simplicity and rapidity—it can be carried out more quickly than staining by Gram's method. Thus a Gram-positive coccus isolated from blood, urine or pus, or the throat etc., which shows neither chaining nor the typical microscopic and colonial morphology of *Staphylococcus aureus* or *albus* can be readily assigned to the genus *Streptococcus* or *Micrococcus*.

This work was carried out with the help of a grant from the Rankin Medical Research Fund, Glasgow University. We should like to acknowledge with thanks receipt of strains from Dr Shattock of the National Institute for Research in Dairying, Dr J. Malcolm and Miss Campbell of the Agricultural College, Glasgow, and Mr F. W. Jelks of the British Postgraduate School, London.

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FURTHER CONSIDERATION OF SOME OF THE FACTORS  
CONCERNED IN INTRACUTANEOUS INJECTION OF CATTLE

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In 1944, Henderson showed the rapidity with which an inoculum reaches the pre-scapular lymph node of the ox after intracutaneous injection of the neck. The smallest volume then injected intracutaneously was 0.5 c.c. and the following opinion (p. 323) was expressed: "When a large dose is given intracutaneously the pressure required for injection no doubt contributes to the rate of absorption but this can hardly apply in the case of the 0.5 c.c. dose". The results of further experiments performed with smaller doses make it necessary to revise this opinion.

*Methods*

The same methods as those described in the earlier report were used to determine the rate of absorption after intracutaneous injection. Briefly these consisted of injection of the skin, slaughter of the cattle at intervals after injection and immediate removal of the regional lymph node for macroscopic examination for evidence of the inoculum having reached the node. Observations were also made on anaesthetised cattle and on dissections of the skin of the neck and underlying tissue after removal from slaughtered cattle.

The inoculum used was india ink, which proved to be more easily detectable in the lymph node in small quantities than a 1 per cent. solution of trypan blue, the inoculum used in the earlier experiments.

*Results*

Ten Devon steers were used for a series of injections of 0.1 c.c. of india ink in the mid-line of the side of the neck about 8 in. cranial to the pre-scapular lymph node. Most of these cattle were injected on both sides of the neck, thus providing two observations. The results, shown in table I, are identical with those obtained for the injection of 0.5 c.c. of a 1 per cent. solution of trypan blue under the same conditions (Henderson).

Four cattle were used for intracutaneous injection on both sides of the neck at a point 1 in. caudal to the base of the ear, that is, about 14 in. from the pre-scapular lymph node. The same rapid involvement of the node was observed (table II).

It has been shown that an intracutaneous injection is almost entirely intralymphatic (McMaster and Hudack, 1935; McMaster and Kidd, 1937; Henderson, 1944) and the results in tables I and II demonstrate conclusively that even with an intracutaneous dose as small as 0.1 c.c. some of the inoculum will reach the regional lymph node in a very short time. In the following experiments an attempt was made to study the mechanism of the rapid flow of the inoculum from the site of injection to the regional lymph node.

In order to eliminate the effect of movement on the rate of absorption, three cattle were anaesthetised by inhalation of chloroform until the corneal and anal reflexes ceased. They were then injected intracutaneously with 0.1 c.c. of india ink at a site 1 in. caudal to the base of the ear, the animal lying on its side with the foreleg drawn back to pull out the folds of skin on the neck. The

skin of the neck of each animal was then reflected and the ink-filled lymph vessels traced towards the pre-scapular lymph node before the animals were slaughtered while still anaesthetised. In each case, within a few minutes of injection, the ink had travelled from 5 to 10 in. but had not reached the node.

TABLE I

*Intracutaneous injection of the neck of cattle; detection of india ink in the pre-scapular lymph node after injection of 0.1 c.c. about 8 in. from the node*

Animal no.	Side Injected	Node removed (seconds after commencement of injection)	Result
C/56 K	Left	27	—
C/58 K	"	40	Trace
C/59 K	"	47	+
C/57 K	"	53	+
C/58 K	Right	58	+
C/61 K	Left	60	+
C/63 K	"	66	+
C/62 K	"	70	—
"	Right	70	+
C/61 K	"	71	+
C/63 K	"	75	+
C/60 K	Left	90	—
C/55 K	Right	99	—
"	Left	129	+
C/60 K	Right	138	+
C/37 J	Left	150	+
"	Right	180	+

TABLE II

*Intracutaneous injection of the neck of cattle; detection of india ink in the pre-scapular lymph node after injection of 0.1 c.c. about 14 in. from the node*

Animal no.	Side Injected	Node removed (seconds after commencement of injection)	Result
C/23 L	Left	48	+
"	Right	55	+
C/22 L	"	120	+
"	Left	130	+
C/24 L	"	150	—
"	Right	150	+
C/25 L	Left	300	+
"	Right	390	+

A number of fresh dissected specimens were used for injection and it was found that an intracutaneous dose of 0.1 c.c. of india ink about 8 in. from the lymph node was sufficient to bring about travel of the ink to the node as an immediate and direct result of the pressure of the injection.

#### Discussion

Although after using 0.5 c.c. doses it had been falsely concluded that the pressure required for intracutaneous injection of such volumes would not contribute to the rate of lymphatic absorption, the statements regarding the rapid involvement of the regional lymph node remain true. From the results of the experiments with dissected specimens there is no doubt that the pressure

required for the intracutaneous injection of 0.1 c.c. is, in itself, sufficient to account for the rapid appearance of the inoculum at the regional lymph node when the distance between the injected site and the node does not exceed 8-10 in. The fact that injections made at a greater distance in anaesthetised cattle failed to stain the node whereas ink injected at the same site in the unrestrained animal reached the node within 48 seconds (table II) suggests that movement of the part may assist further travel beyond the point reached by the effect of pressure. Apparently, when a fluid is injected into the bovine skin, the lymph vessels of the rich cutaneous plexus are damaged. This enables the injected fluid to flow into these vessels through rents in their walls and the presence of valves in the vessels draining the skin directs all flow towards the regional lymph node, so that within seconds of injection much of the inoculum will have left the skin and some may have reached the node. It is of special interest that this occurs after the injection of as small a quantity as 0.1 c.c., a volume which is frequently used in veterinary practice for injection of the skin of the neck of bovines in diagnostic tests, the most important example being the intradermal tuberculin test. The reaction at the site of injection in the sensitised animal is presumably due to some of the tuberculin remaining at this site, but one is forced to conclude from the results of the injection of 0.1 c.c. of india ink that most of the tuberculin has probably left the site of injection within a minute. It is sometimes noted in an animal reacting to the tuberculin test that, on the side of injection, the pre-scapular lymph node is swollen and painful. This involvement of the lymph node has been attributed to the reaction in the neck, but possibly the swollen and painful node is, in itself, a direct reaction to the tuberculin.

### Summary

Experiments are described in which india ink has been demonstrated in the pre-scapular lymph node of the ox within 40-50 seconds of the intracutaneous injection of the neck with volumes as small as 0.1 c.c.

An intracutaneous injection is almost entirely intralymphatic and this rapid flow towards the regional lymph node is largely the result of the pressure required to make the injection.

The possible significance of these observations is mentioned in connection with the intracutaneous injection in veterinary practice of diagnostic agents such as tuberculin.

I am indebted to Mr W. J. Brownsea for his technical assistance. All the observations entailing the killing of cattle were made on healthy animals that had to be slaughtered for other reasons.

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## HÆMOLYSIS IN FRESH BLOOD FILMS

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Fresh dried blood films are received in this laboratory from the wards for morphological examination, unfixed and unstained, as we prefer to do our own staining. Occasionally these were unsatisfactory in that the whole or greater part of the films showed hæmolysis of the red blood cells, a change readily evident to the naked eye, as the normal ground-glass appearance was replaced by clear or translucent areas. The blood films were always prepared by the same technicians, and it was considered worth while to try and find out the cause of the hæmolysis. At first it was thought that hæmolysis occurred during the drying of the film. When a fresh blood film is waved in or above a bunsen flame, immediate hæmolysis occurs if the slide is tilted at an angle to the horizontal. The water vapour produced by the combustion of the gas is sufficient to cause instantaneous hæmolysis, but if the slide is warmed before tilting, no hæmolysis results. This, however, was not the explanation, as it was found that no heat was used in drying the films; the slides were waved in the air until they were dry and were then wrapped in filter paper and sent to the laboratory. As moisture seemed to be the most probable factor in causing the hæmolysis, the effect of moisture on fresh unfixed blood films was investigated directly, with unexpected findings.

If a drop of normal saline is put on a fresh dried blood film the result is immediate hæmolysis. The area of film covered by saline becomes clear and the hæmoglobin from the laked cells is evident as a red deposit in the centre of the drop. Later the hæmoglobin goes to the periphery as the drop dries. Blood films were similarly treated with drops of oxalated plasma and of fresh human serum and in each case immediate hæmolysis resulted. Salt solutions, normal or hypertonic up to a saturated solution of sodium chloride, had a similar effect. It would seem that drying produces irreversible changes in the red blood cells. The alteration is a very rapid one: as soon as the ground-glass appearance is evident in a blood film—usually a few seconds after it has been made—the addition of serum or plasma causes hæmolysis. If plasma is added before drying occurs there is no hæmolysis, the red blood cells float off the slide and behave just like a suspension of red blood cells in any suitable fluid medium. It would appear from this that an aqueous fluid free from fixatives, irrespective of its salt content or osmotic pressure, produces laking of the red blood cells in fresh dried films. Direct examination of such preparations suggests that the process is one of simple solution and not the result of osmosis. At the periphery of the drop of plasma can be seen many cells half in and half out of it. The portion of each such cell in the plasma has lost its hæmoglobin, while the other half, outside the plasma, retains its hæmoglobin. The amount of moisture required for this hæmolysis shows no relationship to the normal water content of the film before it was dried. Indeed, it is possible to show that the water vapour coming from a fresh moist blood film is sufficient to lake the red blood cells of a dried blood film. If a dried blood film is superimposed on a freshly prepared moist blood film but prevented from touching it by small pieces of thin cardboard at either end, and the whole placed under a watch-glass to limit the diffusion of the moisture, the dried film will show a central strip of hæmolysis. The experiment will not succeed if the air is so dry that appreciable drying of the moist film occurs before the two can be placed in apposition, and

in my hands it has been successful only on cold wet days and by the use of a moist film thicker than usual. The moist film also may show areas of hæmolysis particularly in its thinner portions. This is probably the explanation of those areas of hæmolysis found in blood films which are allowed to dry slowly. Such films may be seen when students ignore instructions to dry blood films quickly by waving them in the air, and lay them on a bench to dry; presumably the water vapour coming from the film is not dispersed by air currents, and is available to hæmolyse those red cells which have already dried. The rapidity with which fresh dried red cells will hæmolyse when in contact with plasma or serum may be the explanation of the hæmolysis of blood by repeated freezing and thawing. The freezing might well dry the red blood cells sufficiently to permit of hæmolysis when the melted plasma comes into contact with them on thawing.

In view of these findings the explanation of the laking of the films received in this laboratory was readily understood. The dried blood films were wrapped in filter paper, through which moisture can readily penetrate, and whether they were carried in the hand or the pocket, whether the weather were warm and the bearer's hand perspiring or not, determined the frequency and extent of the hæmolysis. A dried blood film in contact with the dry skin of the hand free from sensible perspiration shows after a minute or two small clear spots of hæmolysis corresponding to the ducts of the sweat glands, and it is evident that insensible perspiration would be adequate to cause hæmolysis in a blood film wrapped in filter paper if held firmly for a considerable time. Since the blood films have been wrapped in ordinary, not filter paper, no further lysed slides have been received.

## BOOKS RECEIVED

### **Lectures on the liver and its diseases**

By H. P. HIMSWORTH. 1947. Oxford: Blackwell Scientific Publications. Pp. xiii and 204; 76 text figs. and one colour plate (4 figs.). 18s. 6d.

This little book on the liver cannot fail to appeal to pathologists, for it is written with vivacity and charm and displays a just appreciation of the outlook of those who spend their lives wondering about the mysteries of disease. There is something in these pages for every type of pathologist whether he be morbid anatomist or histologist, or prefers to think about and plan experiments on the orderly march of tissue reactions. From the very first chapter, the author makes it clear that disease processes are his main theme, illustrating this admirable attitude by considering what happens in the liver when its vascular supply is disturbed, excretory mechanisms are put out of order or the nutrition of the liver cell is interfered with. The logical outcome of such a pathology is the emergence of a clinical mosaic, clearly enough defined for the practical physician, yet allowing a measure of freedom whereby overlap of interrelated tissue disturbance is allowed and a place is reserved for future discoveries. The reader is constantly reminded of the early stages of disease and is encouraged to put into application the lessons which can at present be drawn from these considerations. To cut adrift from the conventional system of rigid clinical divisions requires skill and courage and this is the great merit of Professor Himsworth's lectures. Only in the closing chapters does he appear to falter and the adoption of a formal classification of hepatitis suggests a concession to convention. Time alone will show if the stress placed upon dietary factors in hepatic disease is correct, but that is a minor matter compared with the importance of making people think in terms of cell life and the intrinsic and environmental factors which influence it. The author is to be congratulated on the care he has given to his bibliography, in which adequate attention is paid to British contributions, and on the quality of his illustrations. The Trustees of the Lowell Foundation at Boston deserve the thanks of serious students of liver disease for making these lectures possible, while special commendation should go to the publishers for the high standard of production of the work. Very few errors have been encountered and the index is adequate.

### **The nature and prevention of the cereal rusts as exemplified in the leaf rust of wheat**

By K. STARR CHESTER. 1946. Waltham, Mass.; the Chronica Botanica Co.: London; William Dawson & Sons Ltd. Pp. xi and 270; 12 text figs. \$5.

The group of fungi known as the rusts comprises among its members some of the most destructive plant pathogens. Rust diseases of the various cereal crops cause considerable reduction in yield each year throughout the world, while the losses due to the periodic epiphytotics are estimated at millions of bushels of grain.

Because of their economic importance and mycological interest, the cereal rusts have formed the subject of investigations in all the large grain-growing areas of the world. The need for a comprehensive review of current knowledge is generally recognised. From the title and introduction it would appear that Professor Chester's book was designed to supply such a need. His study is, however, restricted to *Puccinia triticina*, the leaf or brown rust fungus of wheat.

Regarded purely as an assemblage of information on this particular disease, the work is a valuable contribution to our knowledge of the cereal rusts. *P. triticina* occurs wherever wheat is grown. It is of varying importance in different countries, but, on the whole, the evidence bears out the author's contention that, in the world wheat crop, it is second only to *P. graminis* (black or stem rust) in economic significance.

The æcidial (sexual) stage of *P. triticina* has been produced experimentally by several workers on species of *Thalictrum*, the meadow rue. While æcidia have never been found on *Thalictrum* in the wild state, it was assumed, until recently, that this genus functioned as the alternate host in nature. Within the last fifteen years, Russian workers have shown that the æcidia of leaf rust occur in abundance on the basilisk, *Isopyrum fumarioides*, in the Lake Baikal region of Siberia. This would appear to be the only reliable record of the æcidial stage of *P. triticina* in nature.

Like the majority of cereal rusts, leaf rust consists of a large number of physiological races, morphologically alike, but each capable of attacking only certain varieties of the host species. Presumably the races arose, in the first instance, as a result of natural crossing on the æcidial host. It is to be regretted, therefore, that there is, as yet, no information available on the composition of the race flora in Siberia, where the basilisk is a native. In countries where the race flora has been analysed, the appearance of new races is attributed to mutation, or to change in composition of the race population by the selective action of the host varieties. The occurrence of particular races and the severity of the disease are conditioned to a great extent by environmental factors. Hence it is desirable that the methods and cultural conditions employed in race analysis should be standardised.

The ultimate solution to the cereal rust problem lies in the use of resistant varieties. Professor Chester holds that an understanding of the distribution and behaviour of the races is an essential preliminary to breeding for resistance. In consequence, nearly half of the present volume is devoted to physiological specialisation and epidemiology. The two concluding chapters contain a review of theories on the nature of resistance and an account of the breeding work in progress throughout the world.

An authority on cereal disease problems in the southern United States, Professor Chester is well qualified to write on leaf rust. His book will be welcomed by plant pathologists in those regions of the world where the disease is a serious problem. The inclusion of a more general review of all the cereal rusts would have greatly increased the appeal of the book in countries such as Britain, where leaf rust is not a disease of much economic importance.

**The UFAW handbook on the care and management of laboratory animals**

Edited by ALASTAIR N. WORDEN. 1947. London: Baillière, Tindall and Cox. Pp. xvi and 368; 70 text figs. 31s. 6d.

This welcome handbook fills an important place in the library of British laboratory text-books. Its twenty-three chapters present a great deal of practical and expert advice on housing, breeding, feeding and handling the

common laboratory animals. Monkeys, dogs, cats and ungulates are only briefly mentioned along with such rarely employed creatures as shrews, vampire bats and reptiles; but good sources of further information are given. Everything else of importance is fully dealt with, including such less often used animals as the wild mouse, deer mouse, cotton rat, vole, hamster, hedgehog, pigeon and canary. The method adopted in preparing the text was to have each chapter drafted by an expert and to collect supplementary information from answers to a widely distributed questionnaire. The editor then appears to have put the whole together, adding some or all of his own contributions in parentheses. This method of presentation is not ideal since it fails in some chapters to present the information as a coherent whole and involves unnecessarily public amendment of contributors' statements.

### **Symposium on medicolegal problems**

Edited by SAMUEL A. LEVINSON. 1948. Philadelphia, London, Montreal: J. B. Lippincott Co. Pp. xviii and 255; 6 text figs. \$5.

The value of joint meetings between members of the medical and legal professions in the promotion of a better understanding of each other's problems cannot be over-rated. The value of a verbatim record of those meetings, however, is less certain. The present monograph is the record of six discussions of this kind in Chicago. It will doubtless be of interest to those who were present, and to other Americans, unable to attend, but it has little of permanent interest for the foreigner. Inevitably, the circumstances compelled each of the expert opening speakers to use language intelligible to the members of the opposite profession. In "Trauma and Tumors", discussion traversed familiar ground. The discussion on "The medical witness in court" might have been better termed "Perjury by Medical Witnesses". It provides a somewhat startling sidelight on American practice, but no doubt the glaring cases cited will be rare once the Minnesota plan is firmly established. Dr Davidsohn gave a clear and useful account of the application of blood-grouping tests to questions of paternity, and Professor Inbau opened its legal aspect with a particularly snappy story. American methods of evading the rule against self-incrimination in order to admit in evidence the results of tests for alcoholic intoxication are novel: they would be received unfavourably in English courts. The discussion on artificial insemination is of interest sufficiently strong to discount the difficulty experienced in reading an American text.

The monograph is well produced; it is apparently free from typographical errors and there is an adequate index. In these days of acute dollar shortage, prospective purchasers might well make a personal inspection of a borrowed copy before parting with their five dollars.

### **Standard methods of the division of laboratories and research of the New York State department of health**

By A. B. WADSWORTH. 3rd ed., 1947. London: Baillière, Tindall & Cox. Pp. xxxv and 990; frontispiece and 126 text figs. 55s.

This book will be of interest to any one responsible for setting up new departments, particularly of bacteriology. It is true that each man must work out his own salvation, but he may gain some help from studying this extensive and detailed account of the organisation and technical methods of a large and successful group of laboratories. It is clear from this volume that the Americans do not shrink from spending money on

laboratory work or fail to staff, equip and organise a project in proportion to its size.

It is not certain, however, that British readers will experience as much envy as might be expected since our tradition prefers smaller laboratories developing on less formal lines and more often shaped by the influence of a personality or an immediate need than by the blueprints, however generously drawn, of a theoretically perfect service.

The volume itself is beautifully produced on excellent paper, but it is difficult to escape the feeling that much would be gained if critical selection of material were practised and the text reduced to about one-third its present length of over 900 pages.

#### Principles of medical statistics

By A. BRADFORD HILL. Fourth edition, 1948. London: The Lancet Ltd. Pp. xi and 252; 13 text figs. 10s. 6d.

In his preface to this fourth edition, the author refers to the misgivings he experienced as a result of the necessity of repeated reprinting of the third edition without amendment. The fact that he had to yield to such pressure is itself evidence of the wide demand for this book and the useful purpose which it serves.

The main changes now made are additions, and include the interpolation of two extra chapters. Chap. IV—"The Average"—discusses the properties, uses and calculation of the mean, median and mode; chap. XIX, on standardised indices, is an amplification of material which has been excised from the preceding chapter. In other places a discussion of the frequency polygon and histogram has been introduced, in addition to an explanation of the normal curve, and an expanded consideration of pitfalls such as those arising from selection. The new material has been carefully inserted in relation to the existing text with no sacrifice of continuity. We have failed to detect any slips or misprints, but we notice that the author himself has made a numerical correction of the third edition on p. 109.

As is usual in new editions, the book has increased in size, but not greatly; it is still convenient to handle, and it is difficult to see how the existing text could have been shortened without sacrifice of the truly remarkable clarity. This clarity is, of course, the outstanding feature; one does not know whether mathematicians might cavil at over-simplification, but Professor Bradford Hill has succeeded to the full in making statistical methods intelligible to doctors.

#### Gardiner's Handbook of skin diseases

Fifth edition, 1948. Revised by JOHN KINNEAR. Edinburgh: E. & S. Livingstone. Pp. xv and 250; 20 colour plates and 80 text figures. 15s.

This book has reached its fifth edition and yet the author has succeeded in maintaining its original size and object, which is a book for students and general practitioners. This has been achieved by leaving out such subjects as the treatment of syphilis, which is nowadays the sphere of the specialist; this has left room for a detailed description of such practical matters as the treatment of scabies. Recent advances in therapy have been incorporated, though strangely enough no mention is made of the local treatment of sycosis with penicillin. The pictures are apt and of high standard but we feel the histological drawings could be improved upon.

**Handbook of practical bacteriology**

By T. J. MACKIE and J. E. MCCARTNEY. 8th edition, 1948. Edinburgh : E. & S. Livingstone, Ltd. Pp. viii and 624 ; 19 text figs. 25s.

The eighth edition of this popular book appears in a new form because an increase of text has imposed a larger page. In the seventh (1915) edition there were 720 pages, each  $7\frac{1}{4} \times 4\frac{1}{2}$  inches ; in this there are 624 pages, each  $8\frac{1}{2} \times 5\frac{1}{2}$  inches. Additional text given as an appendix in the two wartime editions has now been incorporated in the appropriate chapters. The new material includes an account of Monekton's enrichment method for diphtheria bacilli, but a place has not yet been found for Dubos's cultural methods for tubercle bacilli. The well-known arrangement of earlier editions is retained and the book remains, as it has always been, a truly remarkable combination of systematic and practical bacteriology. It offers the student a sound beginning and the practical worker a clear account of the performance and interpretation of enough reliable methods to let him tackle the routine diagnostic problems of medical bacteriology.

**Corrigendum**

" A text-book of pathology," by E. T. BELL, sixth edition

It is much regretted that an error appeared in our review of this book (this *Journal*, 1947, lix, 738). It was stated that the American price is \$7 (as compared with the English price of 50s.), whereas in fact it is \$10. We offer an unreserved apology to the author and publisher for this serious mis-statement and the implication (in view of the English price) that there had been profiteering on somebody's part.

EDITOR.

# PROCEEDINGS OF THE PATHOLOGICAL SOCIETY OF GREAT BRITAIN AND IRELAND

2nd and 3rd January 1948

The seventy-fifth meeting of the Society was held at the Medical College of St Bartholomew's Hospital, London, on Friday and Saturday, 2nd and 3rd January 1948.

## Communications and demonstrations

Those marked \* are abstracted below

- IWO LOMINSKI. Inhibition of staphylocoagulase by certain antithrombins.
- R. S. ROBERTS. The isolation and purification of a heat-stable toxin from *Bact. coli* cultures.
- C. L. OAKLEY and G. HARRIET WARRACK. The kappa and lambda antigens of *Cl. welchii*.
- M. A. SOLTYS. A case of anthrax in a laboratory worker and observations on the possible source of infection.
- J. CRUICKSHANK and R. F. MENZIES. Luminous bacteria: their reaction on plate cultures to carbohydrates.
- R. D. STUART. The diagnostic importance of urinary antibodies in leptospirosis.
- R. M. FRY and R. I. N. GREAVES. The preservation of bacterial cultures.
- J. W. McLEOD. The epidemiology of diphtheria during the last twenty-five years.
- D. G. FF. EDWARD and V. D. ALLISON. A diphtheria-like disease associated with non-toxigenic diphtheria bacilli.
- I. M. DAWSON and A. S. MCFARLANE. Morphology of vaccinia virus.
- G. L. MONTGOMERY. Involution changes in tubercle follicles during streptomycin treatment.
- L. P. GARROD. The bactericidal action of streptomycin.
- E. M. DARMADY and A. W. BADENOCH. The effects of stroma-free hæmoglobin on the ischæmic kidney of the rabbit.
- B. LENNOX. The large-cell small-acinar thyroid tumour of Langhans and its genesis.
- J. C. HAWKSLEY and G. H. COORAY. The histological study of fragments of gastric mucosa found in aspirated gastric juice.
- \*M. A. M. ABUL-FADL and E. J. KING. The inhibition of acid phosphatases by formaldehyde and its clinical application for the determination of serum prostatic phosphatases.
- C. M. CHU. The action of bacteria on human red corpuscles in relation to their agglutination by various agents.
- D. M. PRYCE. The arteritis of tuberculous meningitis and chemotherapy.
- N. J. BROWN. A case of congenital tuberculosis.
- T. F. HEWER. Lipuria in tigers.
- \*T. D. DAY. The spread of fluids in connective tissue.
- R. J. LUDFORD, J. SMILES and F. V. WELCH. A comparative study of living malignant cells by phase-contrast microscopy and ultra-violet-light photomicrography.



- W. T. ASTBURY, L. DMOCHOWSKI, R. D. PASSEY and R. REED. Electron microscope studies of tissues from high and low breast-cancer strains of mice.
- J. GOUGH and S. E. WENTWORTH. Transparent tissue slices for the demonstration of pneumokoniosis and other conditions.
- G. HADFIELD. The "rheumatic" lung.
- G. J. CUNNINGHAM. Triloculate heart with bilateral pulmonary aneurysm.
- R. A. SHOOTER. Day-by-day variations in normal gastric secretion.
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- A. GLUCKSMANN. Local factors in the histogenesis of hypertrophic scars.
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- W. M. DAVIDSON. Absence of the kidneys as a cause of foetal and neonatal death.
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- D. J. TREVAN and A. B. MACINTYRE. Encephalitis in dogs.
- D. M. PRYCE. (1) Pulmonary arterio-venous angioma. (2) Tracheal bronchus.
- R. E. REWELL. \*(1) Sarcoma of the tibia in a Coypu rat (*Myocastor coypus*).  
 \*(2) Seminoma of the testis in a collared turtle dove (*Streptopelia risoria*).  
 \*(3) Perforation of the palate and destruction of the nasal bones due to nocardiosis in a Bennett's wallaby (*Macropus rufogrisea fruticus*). (4) Multiple gastric ulcers in an anaconda (*Eunectes murinus*).
- A. D. MORGAN and H. A. SISSONS. A case of bone sarcoma supervening on myositis ossificans.
- H. A. SISSONS. Complexity of structure in bone sarcoma.
- K. C. RICHARDSON. Silver impregnation of myoepithelium in thick sections of lactating mammary gland of goat.
- R. J. R. CURETON. Diabetes insipidus associated with hypothalamic granuloma.
- G. J. CUNNINGHAM. Hypertension associated with aberrant renal arteries.
- W. J. HANBURY. Recent additions to the museum of St Bartholomew's Hospital, including:—(1) congenital renal abnormalities, (2) Wilms's tumour after irradiation, (3) infarct of small intestine associated with hypertension, and (4) subacute bacterial endocarditis treated with penicillin.
- R. I. N. GREAVES and R. M. FRY. Some techniques for drying bacterial cultures.
- IAN RANNIE and R. SCHADE. Hemosiderosis following repeated transfusions.
- E. KLIENEBERGER-NOBEL. Capsules and mucoid substances of bacteria in stained preparations.
- J. VALLANCE-OWEN. Histological demonstration of glycogen in necropsy material.
- J. V. DACIE and I. DONIACH. Blood films and photomicrographs illustrating the relationship between siderotic granules and basophilic punctation in red cells.
- J. F. HEGGIE. (1) Large cellular fibroma of lung—pneumonectomy specimen. (2) Colloid carcinoma of urachus. (3) Carcinoma at the umbilicus (? of vitelline duct).
- J. C. CRUICKSHANK. A simple method of testing dye sensitivity of strains of *Brucella*.
- A. H. OAKLEY. The value of large paraffin sections mounted on lantern slides for teaching purposes.

## Abstracts

577.15.082 (acid phosphatase)

## THE APPLICATION OF FORMALDEHYDE TO THE DETERMINATION OF SERUM ACID PHOSPHATASE

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Clinical interest in serum acid phosphatase has increased considerably since Gutman and Gutman (1938) first observed an increase of this enzyme in the sera of patients with metastatic carcinoma of the prostate. The acid phosphatase of the serum is not entirely of prostatic origin, and in doubtful cases, when the increase of the enzyme is not great, a method is required for measuring the fraction which is thought to have originated in the prostate, and contributes to the total elevated serum acid phosphatase.

The alcohol incubation method for distinguishing and measuring the prostatic acid phosphatase (Herbert, 1944, 1945, 1946) is based upon the irreversible destruction of the prostatic enzyme fraction when the serum is incubated with 2/5ths its volume of absolute alcohol. This procedure has been criticised during the course of its use in routine work for not always yielding results which fit the clinical facts. The acid phosphatases of the red corpuscles constitute the greatest source of difficulty in serum acid phosphatase determinations in general and in the alcohol technique in particular. Leakage of these enzymes in considerable amount from the red cells into the plasma or serum can be brought about by the slightest degree of hæmolysis or even without measurable hæmolysis. This factor cannot be practically avoided in the usual way and errors are thus introduced. Other tissue acid phosphatases, *e.g.* those of the bile and kidney, are also affected by alcohol.

The effect of formaldehyde on the various tissue acid phosphatases has been described in a previous communication (Abul-Fadl and King, 1947). Its lack of any action on the prostatic enzyme under certain conditions and its destructive effect on other tissue acid phosphatases and that of the red cells in particular under the same conditions has been demonstrated. Since then, it has been successfully applied in routine determinations of serum acid phosphatase.

The method adopted is based on that of King and Armstrong (1934) as modified by Gutman and Gutman. One ml. of M/50 disodium phenyl phosphate and 2 ml. of citrate buffer (pH 4.95) are pipetted into each of 2 centrifuge tubes, together with 1 ml. of neutral 2 per cent. formaldehyde solution. The tubes are left in the water-bath at 37° C. for 3 min. to allow the contents to attain this temperature; 0.2 ml. of serum is added to one of the tubes and the contents are mixed. The tubes are stoppered and kept in the bath for 60 min. At the end of this time, 1.8 ml. of Folin-Ciocalteu phenol reagent (1 in 3) are added to each tube and 0.2 ml. of serum to the second tube. The 2 tubes are shaken and centrifuged and 4 ml. of the clear supernatants are pipetted into two test-tubes. After the addition of 1 ml. of 25 per cent. sodium carbonate solution, the tubes are replaced in the bath for 15 min. to allow the colours to develop.

The colour intensity is compared with that of a standard solution of phenol and reagent (4 ml.) which has been similarly treated with Na<sub>2</sub>CO<sub>3</sub> solution.

A formaldehyde-stable unit is defined as the amount of enzyme which will liberate 1 mg. of phenol from M/200 phenyl phosphate substrate per hour at 37° C. in the presence of 0.5 per cent. formaldehyde at pH 5.

The effect of formaldehyde on the various tissue acid phosphatases subjected to this procedure may be summarised as follows:—

- (a) The prostatic enzyme, in any amount, is unaffected.
- (b) The red-cell acid phosphatases, when present up to more than 140 units per 100 ml. serum, are almost 100 per cent. inhibited.
- (c) Normal serum and many other tissue acid phosphatases suffer various degrees of inhibition from 40 to 70 per cent.

For ordinary clinical purposes the following limits for the formaldehyde-stable serum acid phosphatase are suggested:—

Normal values	. . . . .	0.3 units per 100 ml. serum
"Suspicious"	. . . . .	3.1-5    "    "    100    "    "
Prostatic	. . . . .	above 5.2    "    "    100    "    "

In most cases the simple formaldehyde technique will serve to distinguish between high acid phosphatases of prostatic origin and those accompanying other conditions. In doubtful cases this can be confirmed with the alcohol incubation method if thought necessary.

A valuable feature of the method lies in providing reliable results even in badly hæmolyzed samples of serum or plasma.

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A. R.

616—018.2

## THE SPREAD OF FLUIDS IN CONNECTIVE TISSUE

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When water or physiological saline was injected into loose connective tissue at a pressure of 10-20 cm. it became localised in the form of an œdematous tumour (Ranvier's Boule d'œdème). In contrast, other fluids such as alcohol, ether, chloroform, xylol and aniline did not show this tendency to accumulate around the point of injection but spread evenly away from it. There was no reason to ascribe this difference in behaviour to surface effects since the rate of flow of water into the tissue was not altered by the addition of soaps or bile salts. Injections of mercury and of air, which do not wet the tissue, also spread rather than localised, but the spread was more uneven. Since the localisation of water could not be explained on purely anatomical grounds, it was suggested that actual combination took place between the water and the tissue. Comparison of the appearance under the dark-ground microscope of freshly excised connective

tissue with that of connective tissue swollen with physiological saline showed that neither the fibres nor the cells in the œdema tumour had increased in size. However, the apparently empty spaces between them had widened considerably. Attention was drawn to the way in which all the structures visible in such preparations moved together when the coverslip was pressed on, just as though they were embedded in some substance of relatively high viscosity. This coherence was as apparent in œdematous as in normal connective tissue. It was concluded that the fibres and cells were embedded in a hydrophilic substance and that localisation of injected water was due to combination with this substance.

Over a period of four days, 5 litres of saline were run through an œdematous tumour measuring  $3 \times 2 \times 0.8$  cm. without any alteration in either naked eye or microscopic appearance. It was considered unlikely that the combination of water with the hydrophilic substance resulted in a solution, since a substance forming a solution would inevitably have been washed out of the tissue by such a procedure. Since it could not be washed out, it was thought possible that the hydrophilic substance might possess some sort of permanent structure. Were such a structure to exist, it would necessarily be least in evidence in the presence of water. On the other hand, withdrawal of water might be expected to reveal it.

Connective tissue was accordingly treated with varying strengths of alcohol and acetone. It was found that, in strengths of alcohol between 50 and 60 per cent. and of acetone between 20 and 30 per cent., the previously empty spaces between the fibres became filled with an opacity which, under the highest magnification, could be resolved into a system of very fine branching fibrils embedded in pellicles of extreme thinness. These appearances only developed when the tissue was freely suspended in the mounting fluid and at the same time was prevented from retracting. These conditions were obtained by tethering the corners of an approximately square lamella of connective tissue with paraffin wax and by irrigation of the under surface of the tissue to prevent its adhesion to the slide. Once formed, the pellicles were permanent structures and did not disappear when the preparation was again mounted in water. They were, however, disintegrated within a few minutes by trypsin. After the action of trypsin, the tissue lost its cohesion; its constituent fibrils instead of maintaining a fixed relationship to one another became free in the surrounding fluid, and were observed to move independently when the coverslip was pressed upon.

The transformation of the hydrophilic substance into visible pellicles and fine branching fibrils in the conditions mentioned could be effected not only by the action of dilute alcohol and acetone but also by protein precipitants such as trichloroacetic acid and mercuric chloride. They also appeared when the tissue was treated with water adjusted to a pH between 3.6 and 3.8. From these facts it was concluded that this substance is a protein.

Pellicles and fine branching fibrils only appeared in tissue treated with strong reagents. It is improbable, therefore, that these structures exist precisely as such in the living body. The fact that they form out of a previously structureless substance is, however, significant. Furthermore, when formed, the other structures such as fibrocytes, collagen bundles and elastic fibres seem unmistakably related to them.

#### *Summary*

Water, in contrast with other fluids, becomes localised when injected into loose connective tissue. This is due to its combining with a hydrophilic substance which lies between the main fibrous components of the tissue and normally joins them together. The substance does not exist in the tissue in the form of an amorphous colloidal solution but possesses a structure which may be revealed by dehydration. Reasons were given for regarding the substance as a protein.

## THE USE OF POLAROGRAPHIC ANALYSIS IN THE INVESTIGATION OF PATHOLOGICAL SERA

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The stimulus to serological research by polarographic methods was given by the work of Purr and Russel (1934) who studied the activating action of blood and serum on certain enzyme systems. They found that blood considerably increased the proteolytic properties of inactivated papain and cathepsin, and that blood from patients with cancer exerted a smaller activating action than did normal blood. They ascribed this activating action of blood to the sulphhydryl groups of the blood proteins. Waldschmidt-Leitz and Purr (1931) established a similar relation between carcinoma and normal sera and attempted to make diagnostic use of this relation.

Brdicka (1933), working in Prague, discovered that sulphur-containing proteins gave characteristic polarographic waves when measured in ammonia buffers containing traces of cobalt salts. Measurement of the height of the wave could be used for the estimation of the quantity of protein. In 1936 Brdicka became acquainted with the work of Purr and Russel and applied his polarographic method to normal and carcinomatous sera. He confirmed the smaller quantity of sulphur-containing proteins in carcinomatous sera. Direct comparison of native normal and native carcinomatous sera showed little difference, but when the serum was given a preliminary treatment with dilute alkali in order to unmask disulphidic groups existing in the interior of the protein molecule, differences between the two types of sera became clear.

An important advance in technique occurred when Brdicka (1938, 1939; Brdicka *et al.*, 1939) demonstrated that filtrates prepared from such alkali-treated sera after precipitation with sulphosalicylic acid showed much greater differences between normal and carcinomatous sera. In this case the difference was reversed, that is, the carcinomatous sera showed higher waves, indicating the presence of more disulphidic substances than in the normal sera. Experimental evidence was brought forward that the serum-filtrate waves are caused by degradation products of serum proteins. These products, though smaller in molecular size than native proteins, are nevertheless of high molecular weight. Although soluble in sulphosalicylic acid solutions they do not pass through cellophane or parchment membranes. All the evidence points to their being proteoses or polypeptides containing cystine nuclei.

Further work showed that a high filtrate wave is not specifically characteristic for cancer sera. They are also often obtained in cases of inflammation. In general the presence of foreign proteins in the blood stream—whether exogenous or endogenous (breakdown of tumour tissue, dispersion of foreign proteins from centres of infection or inflammation etc.)—causes degradation of serum protein and the appearance of large filtrate waves.

Since 1939 over 15,000 sera have been examined by these methods in Prague and the general conclusions drawn from this large quantity of material are that (a) there is an overlap between the upper normal limit for normal sera and the lower limit for early cancer (about 20 per cent. of cases fall into this category), (b) statistically there is correlation between the height of the wave and the degree of advancement of malignancy, (c) on surgical excision of the growth or successful irradiation the wave reverts to normal size, (d) metastases are revealed by a new and gradual increase in wave size, (e) suitably treated cases with inflammations, infections, and other non-carcinomatous disorders show a progressive decrease in wave height.

Owing probably to language difficulties these results of the Czech workers have been largely neglected in this country, but their study suggests that the method might be a valuable, if purely empirical way of assessing objectively the results of therapy in certain pathological conditions. With this object in view some preliminary investigations have been carried out.

The technique for the measurement of the serum filtrate waves has been standardised for use with a Tinsley ink-recording polarograph. 0.4 ml. of blood serum is treated with 1.0 ml. of *N*/10 potassium hydroxide solution and allowed to stand at room temperature for 45 minutes. The proteins are then precipitated by the addition of 1.0 ml. of 20 per cent. sulphosalicylic acid solution. The mixture is allowed to stand for exactly 10 minutes before filtering through a no. 50 Whatman paper. This time must be exactly adhered to, since longer standing in the presence of the precipitate decreases the filtrate wave obtained. The filtrate (0.5 ml.) is added to 5.0 ml. of a buffer of the following composition: *N* ammonia, *N*/10 ammonium chloride, *M*/100 cobalthexamine chloride. In order to render the solution oxygen-free 1.0 ml. of a freshly prepared saturated solution of sodium sulphite is incorporated in 100 ml. of buffer.

Polarograms are run between  $-0.8$  and  $-1.8$  volts. The instrument is set at a sensitivity of 50 micro-amperes. The characteristic wave appears as a rounded maximum having its greatest height at about  $-1.45$  volts. These waves are different in shape from those shown in publications by Brdicka and his collaborators, since the use of *N* ammonia instead of the *N*/10 originally employed by the Czech workers develops rounded maxima instead of double waves.

With this technique, sera from 20 normal males gave waves varying in height from about 4 to 10 micro-amperes. With pathological sera wave heights up to 40 micro-amperes have been obtained (carcinoma or tuberculosis).

In an attempt to ascertain the value of the method in assessing the results of therapy a study of cases of prostatic carcinoma undergoing treatment with synthetic oestrogens is being undertaken. As an example of the results obtained the figures for one case may be quoted.

A polarogram on the serum from this patient on the day preceding the beginning of treatment showed a wave height of 29 micro-amps. Five days after treatment commenced, the wave height was 18 micro-amps. After nine days' treatment the wave decreased to 14 micro-amps. On the 34th day of treatment the wave height was 9.10 micro-amps., that is, just within normal limits.

In view of the fact that certain pathological conditions characterised by large waves are also known to show increased erythrocyte sedimentation rates, a parallel study of the two measurements has been made in both normal and pathological blood. In neither type is there any correlation between the two measurements.

The results so far obtained confirm the view that the method may be a valuable one for assessing the results of therapy in suitably selected cases.

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616.718.5—006.42:599.323.4 (*Myocastor coypus*)SARCOMA OF THE TIBIA IN A COYPU RAT  
(*MYOCASTOR COYPUS*)

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The subject was a male coypu rat, *Myocastor coypus*, the "Nutria" of commerce, which had been in the London Zoo since 7.10.42. In October 1946 a swelling of the upper part of the right hind leg was noticed and the animal was seen to be unwilling to use that leg. On palpation the swelling was found to be attached to the upper part of the right tibia deep to the muscles. Radiography revealed that the bone had been completely replaced by a radiolucent growth for some distance below the epiphysis. There was no evidence that the growth had burst through the periosteum or that new bone had been laid down at its periphery. The periosteum had been stretched, however, and the muscles pushed aside. The appearances were thought to be consistent with the presence of a myeloma, although the rest of the skeleton showed no radiological abnormality. On this assumption, treatment by the implantation of radon seeds was attempted, but the animal died on 26.12.46.

At necropsy on the day of death, the animal was found to be well nourished and with a good coat. The immediate cause of death was an extensive confluent bronchopneumonia which had started to break down in places. The upper end of the right tibia was occupied by a neoplastic mass which had quite destroyed about one half of the length of the bone, but it did not appear to have burst through the periosteum. On section it showed a uniform, greyish-white, soft substance with some firmer trabeculae and a few irregular spicules of bone embedded therein. The whole mass was about 5×3 cm. At its extremities it appeared to have a distinct edge and did not merge into the bone marrow. No abnormalities were found in other bones and no secondary growths in any tissue.

Microscopically the tumour consisted of a mass of collagenous tissue with a number of irregularly arranged, elongated fibroblast-like cells. At the margins, these cells were more abundant and were collected into whorls. The tumour cells showed a high degree of differentiation and were not abundant except at the edges. The surrounding tissues were not invaded, but the tumour, which was large and rapidly growing, had caused extensive destruction of the substance of the tibia. It is best classified, therefore, as a well-differentiated fibrosarcoma.

Fibrosarcomas appear to be quite common in many situations in domestic rodents (Feldman, 1932), but do not appear to have been reported before from this species.

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616 . 681—006 . 46 : 598 . 654 . 3 (*Streptopelia risoria*)SEMINOMA OF THE TESTIS IN A COLLARED TURTLE DOVE  
(*STREPTOPELIA RISORIA*)

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Seminoma of the testis in mammals other than man is well known, and especially in the dog, where the histological appearances are identical with those of the human tumour. They occur also in the horse, bull and other domestic animals (Innes, 1942), although in the horse they are far less frequent than teratomas. In birds they appear to be rare, although Champy and Lavedan (1939) observed similar appearances in regenerating testes. In the case shown, the microscopical appearances were very close to those of the mammalian seminoma, while the tumour undoubtedly arose in a testis.

The bird was an aviary-bred collared turtle dove (*Streptopelia risoria*), of a fancy breed, aged approximately four years, sent in by a private breeder. There was a vague history of indisposition and emaciation for several months before death. At necropsy the body was still fresh and was somewhat emaciated. The only gross abnormality was an ovoid tumour, about  $4\frac{1}{2}$  cm., which occupied the site of the left testis. It bulged forward into the peritoneal cavity, pushing aside the intestine. Its surface was smooth and on gross section a uniform spongy, soft, haemorrhagic structure was found. No secondaries were discovered, but it must be remembered that birds have no lymph nodes.

Histologically the structure, which was uniform throughout five blocks cut, consists of closely packed spheroidal cells, all rather large and well defined, each with a central rounded nucleus and abundant clear cytoplasm. Only a few mitotic figures are seen. The stroma consists of a fine, scanty, fibrous network. It thus resembles very closely the seminoma of mammals.

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616 . 716 . 2—002 . 45 : 576 . 852 . 1 (*Nocardia*) : 599 . 2  
(*Macropus rufogrisea fruticus*)PERFORATION OF THE PALATE AND DESTRUCTION OF THE  
NASAL BONES DUE TO NOCARDIOSIS IN A BENNETT'S  
WALLABY (*MACROPUS RUFOGRISEA FRUTICUS*)

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Nocardiosis may be unfamiliar to many pathologists. It is a specific granuloma due to infection with an organism of the genus *Streptothrix* and resembles human actinomycosis in its morbid anatomy and histology. It is a serious disease of captive wallabies, accounting for 30 per cent. of their deaths (Scott, 1925) or even as many as 47 per cent. (Fox, 1923). The infection usually



starts in the jaw, causing chronic inflammation of the gums and erosion of the mandible and alveolus. It may, however, start elsewhere in the mouth or in the upper respiratory tract, especially the tongue, fauces, palate and nasal cavity. The disease also attacks the lungs and the glandular area of the stomach, from which it may spread to the liver or pancreas. Presumably these sites are secondarily infected from the mouth. Lesions in the lungs are the usual cause of death. The condition is invariably fatal and at the London Zoo we have had no success with either sulphonamides or penicillin.

#### *Case report*

The subject was an elderly male Bennett's Wallaby, *Macropus rufogrisea fruticus*, born in the Gardens on 3rd May 1935. The animal had been running free in a large paddock and no history was available, but usually swelling of one side of the face is noticed for some weeks. The animal died on 2nd February 1947.

At necropsy, the body was still well nourished. The soft palate showed great thickening throughout and when this was incised a caseous mass was revealed which extended up into the nasal cavity and back into the naso-pharynx. When the skull was cleaned by the removal of most of the soft tissues, the process was seen to have caused extensive perforation of the hard palate and destruction of the nasal septum (except its upper part) and turbinate bones. The only lesion in man likely to cause such a condition would be a most extensive gumma, and indeed the two processes bear a certain resemblance. The wallaby, of course, has no soft nasal cartilages. There was no inflammation of the glandular area of the stomach, but both lungs contained many scattered caseous foci.

Sections of the edge of the lesions in the lungs and palate showed a chronic granuloma with a thick layer of fibrous tissue surrounding a caseous centre. In the outer edges of these lesions fungal bodies were seen as short curved rods. In aerobic cultures the fungus forms a branching mycelium, but this is not seen in the tissues.

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## THE INCIDENCE OF PATHOGENIC STAPHYLOCOCCI IN THE THROAT, WITH SPECIAL REFERENCE TO GLANDULAR FEVER \*

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IN the population served by a Royal Air Force general hospital in England, which included the members of many R.A.F. stations in the surrounding country, sporadic glandular fever was a relatively common disease. In the routine examination of throat swabs from cases of pharyngitis due to this and other causes, considerable growths of staphylococci were observed with suggestive frequency from cases of glandular fever. No note of any significant association of this kind was found in the literature. Many authors have stressed the frequent occurrence of Vincent's organisms and of hæmolytic streptococci in this disease; other organisms are only casually mentioned: *Staphylococcus aureus* and *albus*, pneumococcus, *Hæmophilus influenzae*, and diphtheroids. It is interesting to note that staphylococci, if mentioned at all, are regarded merely as "ordinary" organisms of the throat—a view not supported by any accurate observations that I know of.

It seemed worth while, therefore, to investigate the incidence and significance of staphylococci and other organisms in the throat in glandular fever, in other diseases and in health.

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\* The name "glandular fever" is used in preference to "infective" or "infectious mononucleosis", because its historical priority and the advantage of its clinical descriptiveness seem to outweigh the fact that mononucleosis in the blood is a more constant feature than clinically striking glandular enlargement.

*The flora of the throat in glandular fever*

One hundred and forty-eight throat swabs were examined from 66 consecutive sporadic cases of glandular fever; as a control, 597 swabs from 469 consecutive cases of pharyngitis due to other causes were examined with equal care. All swabs were examined by direct smear for Vincent's organisms and by inoculation on horse-blood-agar plates. The great majority were also inoculated on Loeffler slopes and blood-tellurite-agar plates, but the results of these cultures are not considered further. Very few diphtheria bacilli were isolated during this period (none from cases of glandular fever), and diphtheroids were not recorded.

The blood-agar plates were incubated overnight, examined for hæmolytic streptococci and provisionally for staphylococci next morning, and examined finally after a further 24 hours at room temperature, a procedure which was found to help considerably in the identification of staphylococcal colonies, especially if growth was scanty. Representative staphylococcal colonies were tested for coagulase activity by the slide method of Berger (1943). Coagulase-negative staphylococci were not recorded in this investigation. They were in fact surprisingly rare, in contrast to their incidence in the nose. A positive coagulase test was taken as evidence of potential pathogenicity, without regard to pigment formation. In fact a coagulase-positive *albus* strain was isolated only once from the throat in glandular fever and only twice from other forms of pharyngitis. Coagulase-negative *aureus* strains in the throat were equally rare.

The number of the various organisms was noted as scanty (+), moderate (++) or copious (+++). Very sparse growth—up to 5 colonies per half plate for staphylococci—was considered negative. Growth with up to about 50 colonies of staphylococci per half plate were recorded as scanty. A synopsis of the diagnostic laboratory findings in the cases of glandular fever is given in table I. Most cases were clinically obvious, but some of the milder cases presented only the picture of non-specific pharyngitis.

The classification of cases of pharyngitis other than glandular fever (table II) depended on the bacteriological findings, as my only contact with most of these cases was the examination of the throat swab. For this classification, scanty numbers of hæmolytic streptococci or Vincent's organisms were ignored, and only ++ or +++ findings regarded as causative. In this there is an element of inaccuracy, since the finding of numerous hæmolytic streptococci in a sore throat is not absolute evidence that they are its cause, and a negative result for Vincent's organisms certainly does not eliminate this infection, since throat swabs as ordinarily taken may fail to pick up these organisms even if present in large numbers. But such inaccuracies appear unimportant since all the pharyngitis subgroups show a

striking difference from the glandular fever cases in their incidence of pathogenic staphylococci.

TABLE I

*Synopsis of laboratory diagnostic criteria in 66 cases of glandular fever*

Heterophile antibody titre: Paul-Bunnell technique (8 cases)						
Titre . . . . .	1:128	1:512				
No. of cases . . . .	3	5				
Barrett technique* (58 cases)						
Titre . . . . .	<1:40	1:40	1:80	1:160	1:320 and over	
No. of cases. . . .	2	1	6	5	44	
Percentage mononucleosis in blood (61 cases)						
	<50	50-59	60-69	70-79	80-89	90-100
No. of cases	7†	6	9	22	14	3

\* With characteristic absorption reactions, i.e. negative with guinea-pig kidney and positive with ox red cells (Barrett, 1941).

† Some of these cases were first seen only during convalescence.

*Results.* These are summarised in table II. It will be seen that the incidence of pathogenic staphylococci in the throat in glandular fever was almost five times that in the control group as a whole, and more than three times that in any one control subgroup. If only the more profuse growths are considered (i.e. ++ or +++), the disproportion is even greater. These figures, however, record the incidence of staphylococci in cases, not in total throat swabs examined. In many cases, especially of glandular fever, more than one swab was taken, so that the higher incidence of staphylococci in these cases might be due to the greater number of swabs taken. But this possible fallacy may be eliminated by considering only the first swab from each case (see notes to table II); in these the incidence of pathogenic staphylococci was still four times as great in glandular fever as in the whole control group (42.4 against 10.4 per cent.).

This striking frequency of staphylococci in the throat in glandular fever was turned to diagnostic use in this series. Whenever coagulase-positive staphylococci were isolated from a throat swab submitted to the laboratory, the case was further investigated if possible. If the swab had been sent in from an outside station, the station medical officer was asked to send in a blood film and a specimen of blood for titration of heterophile antibody. In 14 of the 66 cases of glandular fever, the diagnosis was originally suggested by a staphylococcus-

positive throat swab. Later, no doubt, most of these cases would have become evident as glandular fever on purely clinical evidence, but some would certainly have escaped diagnosis if the positive throat swab had not suggested the desirability of a differential leucocyte count and heterophile-antibody test. It is notorious that glandular

TABLE II

*Throat flora. Incidence of coagulase-positive staphylococci in 469 cases of pharyngitis from various causes and 66 cases of glandular fever*

Pharyngitis due to	No. of cases	No. showing coagulase-positive staphylococci		Percentage incidence of coagulase-positive staphylococci	
		Total	++ or +++	Total	++ or +++
Hæmolytic streptococci (H.S.)	167	25	14	14.0	8.4
Vincent's organisms (V.)	65	7	4	10.8	6.2
V. with H.S.	36	4	3	11.1	8.3
Negative for H.S. and V.	201	19	13	9.4	6.5
Total	469	55	34	11.7	7.2
Glandular fever	66	35	28	53.0	42.4

## NOTES

*Hæmolytic streptococcal pharyngitis*

Total throat swabs from 167 cases . . . . . 223  
 Total swabs positive for staphylococci . . . . . 32  
 No. of cases with 1st swab positive . . . . . 24 (14.4 per cent.)

*Vincent's angina (including cases of V. plus H.S.)*

Total swabs from 101 (65+36) cases . . . . . 138  
 Total swabs positive for staphylococci . . . . . 13  
 No. of cases with 1st swab positive . . . . . 7 (7.0 per cent.)

*Pharyngitis (negative for H.S. and V.)*

Total swabs from 201 cases . . . . . 236  
 Total swabs positive for staphylococci . . . . . 22  
 No. of cases with 1st swab positive . . . . . 18 (9.0 per cent.)

*Total control group*

Of 469 cases, 49 (10.4 per cent.) had 1st swab positive.

*Glandular fever*

Total swabs from 66 cases . . . . . 148  
 Total swabs positive for staphylococci . . . . . 67  
 No. of cases with 1st swab positive . . . . . 28 (42.4 per cent.)

fever often passes undiagnosed as pharyngitis or Vincent's angina. During an epidemic, mild or atypical cases have a reasonable chance of being detected (Baldrige *et al.*, 1926; Halcrow *et al.*, 1943), but if they appear sporadically they will almost certainly be missed, as the following case-history shows:—

Sgt. R. reported a sore throat. His tonsils showed follicular exudate but no enlargement of lymph nodes was observed, and the diagnosis was acute follicular tonsillitis, probably streptococcal. A throat swab was taken purely as a routine. He was treated with penicillin pastilles and recovered within 48 hours. The throat swab yielded scanty coagulase-positive staphylococci; and because of this a blood film and tube of blood were asked for. The blood film showed 66 per cent. mononuclears, including large abnormal forms; and the heterophile-antibody titre (Barrett method) was 1:320.

In assessing the diagnostic value of a staphylococcus-positive throat swab, however, one must remember its incidence apart from glandular fever. Table II shows that staphylococci were reported in throat swabs from 55 of 469 cases of pharyngitis from other causes. In 37 of these it was possible to do a Barrett test and in many an examination of the leucocyte picture, thereby eliminating glandular fever. These 37 cases must therefore be set against the 14 cases where staphylococci in the throat swab led to a positive diagnosis, indicating a rather better than 1 in 4 chance of correctly diagnosing glandular fever from a positive throat swab alone. This is sufficiently promising to make it worth reporting coagulase-positive staphylococci found on throat-swab plates—not routine practice in most laboratories at present.

The use of the staphylococcus-positive throat swab as a diagnostic measure in the 14 cases referred to above introduces an element of selection into the glandular fever series. If these 14 cases are discarded, 21 of the remaining 52 cases or 40·4 per cent. were staphylococcus-positive. Or, considering only the results of first swabs, 14 or 26 per cent. were staphylococcus-positive—a difference from the incidence of positives in the cases of pharyngitis from other causes which is still highly significant ( $\chi^2 = 11·95$ ). But such rigorous treatment of the selection fallacy itself gives a fallacious impression; since most of the 14 eliminated cases would no doubt have become evident later as glandular fever on purely clinical grounds and would therefore have legitimately joined the series as unselected. So it is fair to say that the incidence of staphylococci in the throat in glandular fever is at least two-and-a-half times (26·7 against 10·4 per cent.) that in pharyngitis from other causes; in fact the ratio is probably nearer 4 : 1.

Table III gives a more detailed analysis of the throat flora in the cases of glandular fever. Since scanty Vincent's organisms and hæmolytic streptococci are common findings in healthy throats, only ++ or +++ findings of these organisms have been recorded. The frequent incidence of both hæmolytic streptococci and Vincent's organisms in glandular fever conforms to the general experience.

#### *The incidence of coagulase-positive staphylococci in healthy throats*

As a possible objection to the general application of these results, it might be suggested that the population which provided them had an unusually high carrier rate for staphylococci, although this is not suggested by the incidence of staphylococci in the control group of other forms of pharyngitis (table II). For a more direct measurement of the carrier rate, however, it was decided to review the incidence of staphylococci in healthy throats in the population concerned.

All the subjects of this study had healthy throats to the extent that they made no complaint of throat symptoms. Some were healthy

individuals whose throats were swabbed because they were possible contacts of diphtheria or scarlet fever; others were hospital patients

TABLE III  
*Cases of glandular fever; analysis of throat flora*

	Cases with pharyngitis		Cases without pharyngitis	Whole series
	Diagnosis suggested by staph. + swab	Others		
Staph. only . . . .	3	7	0	10
Staph. + V.* . . .	7	1	0	8
Staph. + V. + H.S.* . .	2	6	0	8
Staph. + H.S.* . . .	2	7	0	9
V. only* . . . .	0	1	0	1
V. + H.S.* . . . .	0	2	0	2
H.S. only* . . . .	0	7	2	9
Negative all three . .	0	15	4	19
Total . . . .	14	46	6	66
Percentage of cases showing staph.	100	45.7	0	53.0
Percentage of cases showing V.	...	...	...	28.8
Percentage of cases showing H.S.	...	...	...	42.4

\* Only ++ or +++ results recorded for Vincent's organisms and hæmolytic streptococci.

under treatment in medical wards for non-febrile illnesses such as peptic ulcer. In all, the throats of 158 persons were swabbed, and the swabs were examined by the same technique as that used for the cases of glandular fever and pharyngitis. The results are given in table IV. The incidence of coagulase-positive staphylococci was 4.4 per cent., and the growths obtained were mainly scanty.

A 4.4 per cent. incidence in healthy throats does not suggest an unduly high carrier rate in the population concerned, when compared with the rather scanty figures available in the literature. Dingle *et al.* (1944) record an incidence of 7 per cent. for *Staph. aureus* in 94 healthy throats. Miles (1944) states that pathogenic staphylococci are rare in the throat—under 1 per cent.; but he gives no details of the number examined. My finding falls between these two figures.

Table IV also records the throat flora of 36 patients with burns, none of whom complained of sore throat. Swabs were taken as a routine from unselected cases admitted to the hospital burns centre. They have not been included with the main control group because most of them had a staphylococcal infection of the burnt areas, and

this might be expected to raise the incidence of staphylococci in their throats above normal, an expectation that was confirmed, since the

TABLE IV  
*Flora of healthy throats*

	158 persons (infectious disease contacts, etc.)	36 cases of burns
Coagulase-positive staph. + . 4	7 = 4.4 per cent.	2
" " " ++ 3		4
" " " +++ 0		0
Vincent's organisms* . . . 1		0
Hæm. strept.* . . . . 22 = 13.9 per cent.		3 = 8.3 per cent.
Negative for all three . . . 132 = 83.5 per cent.		30 = 83.3 per cent.

\* ++ or +++.

incidence of pathogenic staphylococci in the throats of this group was 16.7 per cent., or four times that in the main group of 158 controls. Statistical analysis showed that the difference was significant ( $\chi^2 = 5.20$ ).

#### *Incidence of staphylococci in the nose*

In the later part of this investigation, nasal swabs were taken where possible at the same time as the throat swabs. Table V shows the results. The numbers are small, and the different incidence of nasal coagulase-positive staphylococci among the various groups of cases is probably not significant. But the incidence in the whole series—37.0 per cent.—indicates that this particular population did not show any specially heavy carrier-rate for staphylococci. The figures of other workers for the nasal incidence of pathogenic staphylococci in adults are similar to mine (39 per cent., Hallman, 1937; 34 per cent. and 42 per cent., McFarlan, 1938; 47 per cent., Miles *et al.*, 1944).

There is an obvious positive correlation between the incidence of pathogenic staphylococci in throat and nose. The numbers in each group are admittedly small; but all groups show correlation of positive sign and, omitting the very small group of "healthy throats—burns", of approximately the same degree. Considering all cases together, 31 of 58 or 53 per cent. of the throat-positive cases were nose-positive, and 33 of 115 or 28 per cent. of the throat-negative cases were nose-positive; the correlation is statistically significant ( $\chi^2 = 9.09$ ).

It is interesting to note that the incidence of pathogenic staphylococci in the nose is not significantly higher in glandular fever than in the control groups, in striking contrast to the incidence in the throat. It is also of some interest that in the nose coagulase-negative



staphylococci, almost all *albus*, were found almost as frequently as coagulase-positive strains, whereas in the throat coagulase-negative strains were rare in all the groups examined.

TABLE V

*Incidence of coagulase-positive staphylococci in the nose*

Case groups	Nose		
	Positive	Negative	Percentage positive
Glandular fever *			
Throat positive . . . 17 (23)	9 (14)	8 (9)	53.0 (60.9)
„ negative . . . 23 (17)	7 (5)	16 (12)	30.4 (29.4)
Total . . . . . 40 (40)	16 (19)	24 (21)	40.0 (47.5)
Other forms of pharyngitis			
Throat positive . . . . 22	11	11	50.0
„ negative . . . . . 35	8	27	22.9
Total . . . . . 57	19	38	33.3
Chronic tonsillitis			
Throat positive . . . . 14	7	7	50.0
„ negative . . . . . 13	3	10	23.1
Total . . . . . 27	10	17	37.0
Healthy throats			
Throat positive . . . . . 3	2	1	66.6
„ negative . . . . . 34	11	23	32.4
Total . . . . . 37	13	24	35.1
Healthy throats: burns			
Throat positive . . . . . 2	2	0	100.0
„ negative . . . . . 10	4	6	40.0
Total . . . . . 12	6	6	50.0
Series as a whole			
Throat positive . . . . . 58	31	27	53.0
„ negative . . . . . 115	33	82	28.0
Total . . . . . 173	64	109	37.0

\* The figures in brackets record the results obtained by considering all the swabs taken; in most of the glandular fever cases repeated swabs were taken from both nose and mouth. For comparison with the other groups of cases, in the majority of which only single swabs were taken, the unbracketed figures give the results for the first nasal swab and the throat swab taken nearest to it in time, usually the same time.

### *Throat flora in chronic or recurrent tonsillitis*

After I had begun to inquire as closely as possible into cases in which staphylococci were isolated from the throat swab, I was often told of patients with repeated sore throats. This suggested that the chronically inflamed tonsil might often either be infected by or carry staphylococci, a suggestion that agrees with the work of McAuliffe and Leask (1941), who isolated *Staph. aureus* from the tonsils of 14

of 15 patients with chronic tonsillitis. However, to define the incidence of staphylococci in such cases among the population served by the hospital, 36 patients admitted to the hospital for tonsillectomy because of recurrent sore throat were investigated. Throat swabs were taken just before operation and smears and cultures were taken from the crypts of the excised tonsils. The surface swabs (table VI) gave an

TABLE VI

*Throat flora in 36 cases of chronic tonsillitis*

Throat flora	Throat swab immediately before tonsillectomy (31 cases)	Smears and cultures from crypts of tonsils after removal (36 cases)
Coagulase-positive staph. + . . .	2	5
" " " ++ . . .	3	4
" " " +++ . . .	1	10
6 = 19.4 per cent.		19 = 52.8 per cent.
Vincent's organisms * . . .	1	12
Hæm. strept.* . . .	3	15
Negative for all three . . .	21	7

\* ++ or +++.

incidence of coagulase-positive staphylococci of 19.4 per cent.—more than four times the incidence (4.4 per cent.) in healthy throats,\* though much lower than in McAuliffe and Leask's cases, and higher, though not greatly so, than in any of the groups of acute pharyngitis, other than glandular fever, described in this paper. The cultures from the crypts of the tonsils showed a still higher incidence of staphylococci—over 50 per cent. Obviously, therefore, as judged from the surface swab results, chronically or repeatedly inflamed tonsils harbour pathogenic staphylococci much more often than normal throats.

This association of staphylococci with chronic or recurrent tonsillitis raises the possibility that the high incidence of staphylococci in glandular fever throats might be due to an association between chronic tonsillitis and glandular fever: an individual with a chronically infected throat might be unduly susceptible to glandular fever, and the staphylococci in the throat might merely reflect the pre-existing chronic tonsillitis, with the organisms perhaps brought to the surface of the tonsils by the superadded acute exudative inflammation produced by the glandular fever. However, in 37 of the cases of glandular fever the patient was carefully questioned about previous sore throats and colds. In the absence of control observations it is not possible to say whether these patients showed a significantly raised incidence of such infections, but table VII shows that the

\* Statistical analysis shows that this difference is significant;  $\chi^2 = 6.83$ .

incidence of staphylococci in the throat was as high in the patients who had not been subject to sore throats and colds as in those who had.

TABLE VII

*Incidence of history of previous throat or nose infection in  
37 adequately questioned cases of glandular fever*

History	No. of cases	No. positive for staphylococci
Not subject to sore throat or colds	18	11
Not subject to sore throat: frequent colds or chronic nasal catarrh	5	1
Subject to sore throats		
? slightly . . . .	7	4
definitely . . . .	7	3

*The significance of throat staphylococci in glandular fever*

There are three possible interpretations of the relatively high incidence of pathogenic staphylococci in the throat in glandular fever: (1) the staphylococci might be the causal organism of the disease; (2) they might represent a true secondary infection, i.e. their presence might contribute to the symptomatology of the disease; or (3) they might be commensals, taking root in a soil prepared by the true virus of the disease.

The first possibility is inherently unlikely. No causative agent has as yet been satisfactorily isolated, but the general picture of the disease strongly suggests a filterable virus, as does its obvious kinship to infective hepatitis and rubella. The question remains whether the staphylococci constitute a true secondary infection, or are merely favoured commensals. No significant difference in the appearance of the throat was noted in the present series between cases with and without staphylococci. However, an attempt was made to decide the question by estimating the staphylococcal antitoxin content of the serum in a series of cases. The method used was essentially that of Parish *et al.* (1934), with some of the modifications of Lichty *et al.* (1943). There is general agreement in the literature that the average antitoxin level in normal people is about 0.75 international units per c.c. (Murray, 1935; Dolman, 1935; Blair and Hallman, 1935-36; Whitby, 1936; Lichty *et al.*, 1943; Seeley *et al.*, 1944); and that 2.0 units per c.c. is the upper limit of normal. Only Seeley *et al.* record slightly higher levels, and they in only 2.6 per cent. of their series. It is significant, therefore, that in 3 of 24 glandular fever cases with staphylococcus-positive throats I obtained titres above this level—values of 8, 3 and 3 units per c.c. The case with the high titre of 8 units showed a steadily falling titre after recovery from the attack of glandular fever, a definite indication of staphylococcal

toxæmia during the attack. The remaining staphylococcus-positive cases and the 14 staphylococcus-negative cases tested showed values within normal limits and a normal mean value; which, however, does not exclude the presence of a true staphylococcal infection.

### DISCUSSION

During this work I formed the impression that the presence of pathogenic staphylococci in the throat has some significance and that the significant association can often be identified. Three such associations emerge, namely with glandular fever, chronic or recurrent tonsillitis, and staphylococcal infections of the skin, as in burns and other lesions.

The significance of the throat staphylococci in glandular fever has already been partly discussed in earlier sections of this paper. They are apparently secondary invaders, either as commensals or at least in some cases as pathogens. Their most obvious source is the nose, a common site of staphylococcal carriage, but since the strains were not typed this cannot be definitely established. The facts, however, are highly suggestive. There is a positive correlation between the incidence of staphylococci in the nose and in the throat in glandular fever as well as in the other groups studied. This might mean that both sites tended to become infected from a third source; but the fact that in glandular fever the throat incidence rises strikingly whereas the nasal incidence is not significantly raised suggests that the throat, collectively regarded, is being infected from the relatively static reservoir of staphylococci in the nose. An appreciable number of throat-positive cases gave negative cultures from the nose, some of them repeatedly. In these cases the nose may have been positive at first and become negative after infecting the throat; but this seems unlikely in view of the demonstration by Miles *et al.* that the staphylococcal nasal carrier state tends to be persistent. It may be, therefore, that in these cases the throat was infected either from the skin—a fairly common carrier site, 18·4 per cent. of healthy people according to Miles *et al.*—or from some exogenous source.

Whatever the source of the infection, it would appear that the pharyngeal inflammation in glandular fever may specifically prepare the soil for staphylococcal settlement. It may be significant that another virus, influenza, appears to prepare the lung for staphylococcal infection; various workers have reported staphylococcal pneumonia in influenza epidemics—a type of pneumonia not commonly found apart from influenza (Chickering and Park, 1919; Patrick, 1923; Finland *et al.*, 1942; Michael, 1942; Guthrie and Montgomery, 1947).

The significance of the high incidence of pathogenic staphylococci in chronically inflamed tonsils is not clear. Such tonsils apparently form a good nidus for growth not only of staphylococci but also of hæmolytic streptococci and Vincent's organisms. To what extent the

staphylococci contribute to the inflammation of the tonsil is not known.

The third association is of pathogenic staphylococci in the throat with staphylococcal infection of the skin. This is shown by the considerable rise in the incidence of pathogenic staphylococci in the healthy throats of persons with burns (table IV). And it is perhaps significant that among the staphylococcus-positive cases of pharyngitis other than glandular fever four were patients with skin lesions infected by staphylococci. There is no reason to suppose that the skin infection is secondary to that in the throat; in the burns cases the incidence of staphylococcal infection was much higher in the burns than in the throat. In these cases, therefore, the throat is presumably infected from the skin.

### SUMMARY

The bacterial flora of the throat in glandular fever was investigated. Pathogenic staphylococci were isolated in appreciable numbers from the throats of 53 per cent. of 66 sporadic cases occurring among members of the Royal Air Force.

The incidence of pathogenic staphylococci in the throats of a control group of 469 cases of other forms of pharyngitis was 11·7 per cent. and in 158 healthy throats 4·4 per cent. The differences, after allowing for fallacies of selection, are statistically significant.

Serum antitoxin estimations indicate that in glandular fever the staphylococcus may sometimes contribute to the symptoms of the disease.

The isolation of pathogenic staphylococci from a throat swab may be helpfully suggestive of the diagnosis in pharyngitis due to unsuspected glandular fever.

Pathogenic staphylococci were isolated from the crypts of 53 per cent. of the tonsils removed from 36 cases of chronic or recurrent tonsillitis.

Infected burns, and probably other staphylococcal infections of the skin, raise the incidence of pathogenic staphylococci in the throat.

A positive correlation was found between the nose and throat incidence of pathogenic staphylococci in the various case groups investigated. It would appear that the throat may become infected either from the nose or from the skin.

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# UREASE AND OTHER BIOCHEMICAL REACTIONS OF THE PROTEUS GROUP

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THE investigation of colonies of non-lactose-fermenting organisms, later proved to belong to the *Proteus* or paracolon groups, plays a large part in the routine bacteriological examination of faeces. The increased use of liquid enrichment media in routine laboratories is particularly responsible for the frequency with which *Proteus* is encountered, for most strains grow freely in such media and on desoxycholate-citrate agar, a useful medium for sub-culture. Considerable time may be spent over the biochemical and serological investigation of non-lactose-fermenting colonies before they are identified as belonging to the *Proteus* group and eliminated as specific pathogens. Although the majority can be recognised by their ability to swarm on agar, difficulty may be caused by many of the remainder because of the similarity of their biochemical reactions to those of certain *Shigella* organisms. Any method which helps in the identification of *Proteus* strains should prove of value to the routine bacteriologist. With this aim in view, it was decided to investigate the urea-splitting properties and certain biochemical reactions of all *Proteus* cultures isolated during the routine examination of faecal specimens in this laboratory.

It has been known for a number of years that members of the *Proteus* group can hydrolyse urea. All the 194 *Proteus* cultures studied by Moltke (1927, p. 91) attacked urea and this property has recently been more fully investigated by Rustigian and Stuart (1941), Stuart, van Stratum and Rustigian (1945) and Christensen (1946). These workers also studied the urease activity of paracolon bacilli, which they classify on the basis of their biochemical reactions into three groups resembling the *Escherichia* (= *coli*), intermediate and *Aerobacter* (= *aerogenes*) divisions of the coliform group. Urease-positive strains were found only among the intermediate and *Aerobacter* groups and these could be distinguished from *Proteus* organisms by the rate of hydrolysis and the use of suitably buffered media.

In the present investigation it was decided to compare two urea media referred to as A and B. In both, phenol red was used as an



indicator and hydrolysis of urea was detected by the appearance of a red-violet colour due to the increased alkalinity produced by liberation of ammonia from urea. Medium A was a highly buffered solid medium of poor nutritive quality based on a similarly constituted liquid medium described by Rustigian and Stuart (1941). With a medium of this type hydrolysis of urea within 48 hours is confined to the *Proteus* group. Ferguson and Hook (1942-43) obtained positive results on the liquid medium with 34 of 35 *Proteus* organisms, and all of 70 *Salmonella* strains were urease-negative.

Medium B was originally designed by Christensen. It was less strongly buffered than medium A and supplied additional nitrogen and carbon in the form of small amounts of peptone and glucose. Christensen found that 21 paracolon strains of the *Aerobacter* and intermediate groups gave a positive result on this medium, although most of these required several days to show a moderately strong reaction compared with 1-6 hours for *Proteus* strains. He also tested a number of *Salmonella*-*Shigella* cultures on the same medium over a period of 6 months with consistently negative results. Similar negative findings with intestinal pathogens were reported by Ewing (1946).

## METHODS

### *Selection of cultures for investigation*

Fæces were inoculated as a routine directly on to desoxycholate-citrate agar and Wilson and Blair's medium. A broth suspension of fæces, if solid, or the untreated fæces, if liquid, was also inoculated into Kauffmann tetrathionate broth and into selenite-F, from each of which a loopful was plated out after 18 hours' incubation on to desoxycholate-citrate agar and Wilson and Blair's medium. Rectal swabs were usually received only from cases of suspected bacillary dysentery and Wilson and Blair's medium and tetrathionate broth were then omitted. Although *Proteus* strains produce fairly characteristic appearances on Wilson and Blair's medium, only colonies suspected of being *Salmonella* were investigated. For this reason isolation of *Proteus* cultures was not systematically undertaken from these plates, though two strains isolated only on Wilson and Blair's medium are included in this series. Non-lactose-fermenting colonies were usually picked for further investigation into a tube of peptone water containing a lead acetate paper, on to an agar slope and into certain sugar media. A MacConkey plate was inoculated from the same colony to check the purity of the inoculum. For the purpose of this investigation all non-lactose-fermenting organisms which produced acid, with or without a bubble of gas, in glucose, mannitol, sucrose, maltose or salicin within 48 hours were inoculated on to a urea slope (medium B), irrespective of their ability to spread or to produce hydrogen sulphide or indole. Cultures giving a positive reaction within 6 hours were provisionally regarded as *Proteus* and were sub-cultured on to Dorset's egg medium and kept in the refrigerator for full investigation.

*Proteus* strains isolated on more than one medium from the same specimen and having similar biochemical reactions were regarded as identical and only one strain was selected for further investigation. When apparently similar strains were isolated from more than one specimen from the same patient, only one, usually the first, was investigated.

*Method of inoculation and biochemical tests*

All urease-positive cultures were subjected to the following biochemical tests in batches of 24-36, usually within 3 months of isolation.

Inoculations were made from a 24-hour agar-slope culture over the surface of one tube of medium A and one of medium B, and also into peptone water. The cultures were incubated at 37° C. and the urea tubes were read at 3, 6, and 24 hours after inoculation and, if negative, daily for 7 days. A slight pink colour on the surface of the medium was recorded as a trace reaction, diffusion of colour into the butt as a moderate reaction and complete diffusion throughout the medium as a strong reaction. The peptone-water culture was incubated for 24 hours and 2 or 3 drops from a 50-dropper pipette were then added to the following media: peptone water containing a lead acetate paper, glucose, mannitol, maltose, lactose, sucrose, salicin, glucose MacConkey broth and two tubes of glucose-phosphate medium. In addition, Koser's citrate medium and a gelatin stab were inoculated with a straight wire.

*Urea media.* Medium A contained the ingredients described by Rustigian and Stuart with the addition of 2 per cent. agar. Medium B was prepared according to the directions of Christensen, apart from a slightly shorter time of sterilisation. The detailed method of preparation of these media is given in the appendix.

*Hydrogen sulphide.* This was indicated by the blackening of a strip of filter paper soaked in a saturated solution of lead acetate and placed over a 2 per cent. peptone water (Eupeptone) culture. Readings were made after 24 hours' incubation and cultures failing to produce hydrogen sulphide were subsequently re-tested using tryptic digest broth instead of peptone water. Blackening confined to the edge of the paper was recorded as a negative result.

*Sugar media.* A solution consisting of 1 per cent. sugar, 1 per cent. peptone and 0.5 per cent. sodium chloride was distributed in 5 ml. quantities in test-tubes containing Durham tubes and autoclaved at 10 lb. for 15 minutes. The presence of acid and gas was recorded after 1, 2, 10 and 21 days' incubation.

*Glucose MacConkey broth.* The method described in the Ministry of Health Report (1939) for the preparation of single-strength lactose MacConkey broth was followed in making up this medium, with the substitution of glucose for lactose. The tubes were incubated for 48 hours at 44° C. in a water-bath and the presence of growth, with or without gas production, was then recorded.

The indole, methyl-red (M.R.), Voges-Proskauer (V.P.), and citrate tests were carried out in the usual way. Gelatin stabs were examined for liquefaction at 6 and 21 days after standing at bench temperature. All other cultures except the glucose MacConkey broth tubes were incubated at 37° C.

## RESULTS

During the period of this investigation (October 1946-July 1947) approximately 1050 faecal specimens were examined, of which only a few were rectal swabs. It is impossible to state exactly how many of these were repeat specimens from any one case, but their number is estimated as at least 10 per cent. Most specimens were from acute or convalescent cases of gastro-enteritis. In this series 120 strains which strongly attacked urea were isolated from 115 different patients, excluding similar strains isolated more than once from the same case. Two strains with different biochemical reactions were isolated from 4 cases on separate occasions and in one case from a single specimen. Urease-positive organisms were probably isolated from at least 12 per

cent. of patients from whom specimens were examined. The percentage of *Salmonella-Shigella* isolations among these 115 specimens was 8.7, compared with 8.6 among the total faecal specimens examined during the period of the investigation.

Rustigian and Stuart (1945) suggested the following division of the *Proteus* group based on biochemical reactions: *P. vulgaris*, *P. mirabilis*, *P. morgani* and *P. rettgeri*. The merits of this classification are discussed later, but it provides a convenient means of referring to collections of strains with similar biochemical reactions and has been followed in this paper. The 120 urease-positive cultures undergoing investigation were grouped as follows: *P. vulgaris*, 14 strains; *P. mirabilis*, 86 strains; *P. morgani*, 9 strains; *P. rettgeri*, 11 strains. One-hundred-and-seven *Proteus* strains were isolated from specimens inoculated both directly and indirectly on to desoxycholate-citrate agar. Table I compares the frequency with which these isolations

TABLE I

*Comparison of direct and indirect plating on desoxycholate-citrate agar in the isolation of 107 Proteus strains*

Sub-group	No. of strains isolated	Direct plating	Sub-culture from tetrathionate broth	Sub-culture from selenite-F
<i>P. vulgaris</i> . .	13	6	8	9
<i>P. mirabilis</i> . .	75	29	68	41
<i>P. morgani</i> . .	8	6	2	2
<i>P. rettgeri</i> . .	11	2	10	8
Total . .	107	42	88	60

were made from different media for the various sub-groups; although the numbers are small, the results suggest that *morgani* strains are less able to utilise the enrichment media. It is also of interest to record that at the time of isolation as many as 33 of 119 cultures (27.7 per cent.) did not spread on a 2 per cent. agar slope. These include all 11 strains of *P. rettgeri*.

### *Urease activity*

The majority of strains gave a trace or moderate reaction on medium A after 6 hours' incubation and a strong reaction at 24 hours. In 9 cases (*P. mirabilis* 8, *P. vulgaris* 1) this was delayed for 48 hours, but only one strain (*P. rettgeri*) failed to attack urea within that time. This strain produced a moderate reaction after 7 days' incubation. With medium B very rapid results were obtained, a fact of some importance in routine diagnostic work. All the 120 strains gave a moderate or strong reaction within 3 hours and a strong reaction at 6 hours; in all of many cultures examined after 1½ hours' incubation a moderately strong reaction was already apparent.

In addition, the urease reaction of 65 paracolon strains was investigated and the results were correlated with the indole, M.R. and V.P. reactions and with growth in glucose MacConkey broth and citrate. All these strains were urease-negative on medium A, but 8 gave a moderate reaction on medium B after 24 hours' incubation. These 8 strains were all able to utilise citrate; this result confirms the findings of Christensen, who noted that urease activity among paracolon bacilli was limited to the *Aerobacter* and intermediate groups.

### Biochemical reactions

The results of the biochemical reactions for each sub-group, summarised in table II, are in accordance with those reported by

TABLE II

Summary of biochemical reactions of 120 *Proteus* strains

Sub-group	No. of strains tested	Days of incubation of sugars	Acid with or without bubble of gas						Glucose MacConkey at 44° C.		H <sub>2</sub> S+		Indole +	M.R. +	V.P. +	Citrate +	Gelatin liquefied
			Lactose	Glucose	Mannitol	Maltose	Sucrose	Saltin	Acid	Acid and gas	Peptone water	Broth ‡					
<i>P. vulgaris</i>	14	1-2 3-21	0 0	14 14	0 0	14 14	10 10	11	2	11	3	14	14	0	3	14	
<i>P. mirabilis</i>	86	1-2 3-21	0 0	86 * 86 *	0 0	0 0	0 85	0 13	3	83	76	10	2	84	14	39	86
<i>P. morgani</i>	9	1-2 3-21	0 0	9 9	0 0	0 0	0 6	0 4	0	0	0	9	9	9	0	0	0
<i>P. rettgeri</i> †	11	1-2 3-21	0 0	11 11	1 11	0 0	0 8	7 8	0	0	0	0	10	11	0	11	0

\* Includes three anaerogenic strains.

† All strains were anaerogenic.

‡ Only strains negative in peptone water were re-tested in broth.

previous workers. A bubble of gas was produced in glucose by 83 of 86 *mirabilis*, all of 14 *vulgaris* and 3 of 9 *morgani* strains. Gas production in other sugars was variable, though a small amount was usually given by *P. vulgaris*. Most of the *rettgeri* strains took several days to ferment mannitol and all of them were anaerogenic. At 44° C. gas was produced by all the aerogenic *mirabilis* strains though by only two *vulgaris* strains. None of the *morgani* or *rettgeri* strains grew at this temperature.

Hydrogen sulphide, if produced, was usually abundant; the significance of the results with *P. morgani* is discussed later. The M.R. and V.P. reactions were usually weak and difficult to interpret, though the indole reaction, if positive, was particularly marked. Most of the organisms which grew in Koser's citrate and which

liquefied gelatin did so within 2 and 6 days respectively. Most cultures behaved consistently throughout the range of the biochemical reactions tested and there was usually no difficulty in assigning them to their correct sub-group.

### *Swarming and pigment production by P. rettgeri*

All of 11 *rettgeri* cultures were found to be non-motile on first isolation and an attempt was made to produce motility and swarming in these strains. After several daily sub-cultures in broth the cultures were transferred to Craigie tubes containing 0.15 per cent. agar. After two or three passages in this medium, 0.3 per cent. agar Craigie tubes were inoculated and subsequently broth cultures. All 11 cultures were then motile when examined in a hanging-drop preparation and 10 were induced to spread on 1 per cent. agar. Plates of 9 cm. diameter were inoculated in the centre and incubated at approximately 34° C. and at bench temperature. After two days 100 per cent. of the surface had been covered by 4 strains, 75 per cent. by 3, 50 per cent. by 2 and 25 per cent. by 1 strain, the higher temperature being the more favourable.

A feature common to all 11 *rettgeri* strains was the pigmented nature of the colonies on desoxycholate-citrate agar. After 24 hours' incubation the colour varied from pale to bright yellow, but at 48 hours the colonies had increased in size and showed a well differentiated orange-yellow centre surrounded by a pale translucent periphery. Two strains also produced a brownish pigmentation on nutrient agar which readily diffused through the medium. Pigment formation was increased by allowing the cultures to stand at bench temperature.

### DISCUSSION

The inclusion of a urea medium in the routine investigation of intestinal organisms has been recommended by a number of authors (Ferguson and Hook; Ewing; Christensen). The results of this investigation have confirmed the fact that a highly buffered medium such as medium A, though of value as a means of distinguishing members of the *Proteus* group from other non-lactose-fermenting organisms, is less suitable than medium B for the rapid identification of *Proteus* strains and the detection of urease-positive paracolon bacilli. Medium B has been used in the routine bacteriological examination of faeces in this laboratory for more than 18 months and has proved particularly valuable in the investigation of non-lactose-fermenting colonies obtained on primary and indirect culture and of non-spreading strains giving fermentation reactions suggestive of the *Proteus* group. Inoculation of the medium with a *Proteus* culture or even a single colony will cause a demonstrable change of pH after one or two hours in the 37° C. water-bath; in some instances

a heavy inoculum produced a detectable pink colour within a few minutes. Using measured volumes of bacterial suspensions and a highly buffered medium Stuart *et al.* showed that the speed of urease production was considerably slower with *P. morgani* than with other *Proteus* strains. That this difference was not observed by Christensen or in this investigation is probably due to the use of a less strongly buffered medium and a heavier inoculum. The medium has also proved of value in the identification of paracolon bacilli, some of which give a positive urease test after overnight incubation. *Salmonella* and *Shigella* cultures have been reported as consistently urease-negative (Christensen; Ewing) and similar findings were obtained with a number of pathogenic strains tested during this investigation.

Certain observations regarding hydrogen sulphide production by *morgani* strains are of interest in view of the discrepant findings previously reported (Rauss, 1936; Rustigian and Stuart, 1945). All our strains of *P. morgani* failed to blacken a lead acetate strip in 24 hours when grown in 2 per cent. peptone (Eupeptone) water, but did so when grown in digest broth. Similar observations were made with 3 *vulgaris* and 10 *mirabilis* strains. These findings emphasise the superiority of digest broth over peptone water as a substrate for this test, though results might have been improved by changing the brand or increasing the concentration of peptone.

Rustigian and Stuart (1943) described a group of paracolon bacilli which strongly attack urea and ferment mannitol but not maltose. They considered that the urease activity of these organisms together with their ability to swarm under suitable conditions was sufficient evidence for including them in the *Proteus* group (*P. rettgeri*). Their main reactions are rapid fermentation, usually anaerogenic, of glucose and frequently of salicin, slower fermentation of mannitol and sucrose, production of indole, growth in Koser's citrate, rapid hydrolysis of urea and ability to swarm on 1 per cent. agar. Hydrogen sulphide is not produced, gelatin is not liquefied, and there is no growth at 44° C. in glucose MacConkey broth. Eleven of the strains investigated showed these characteristics though one failed to produce indole and another, though motile, did not spread. Similar biochemical reactions including marked urease activity were given by a culture of *Bacterium rettgeri* received from the National Collection of Type Cultures (no. 1501) (St John-Brooks and Rhodes, 1923). Attempts to induce this strain to swarm were unsuccessful, though good motility was produced at bench temperature. Pigmented colonies on desoxycholate-citrate agar were also obtained.

For 50 years after Hauser's original classification of *Proteus* in 1885, gelatin liquefaction, swarming on solid media and hydrogen sulphide production were regarded as essential criteria of the *Proteus* group, though non-motile strains had been recognised (Felix, 1922-23; Moltke, 1927, p. 43). General acceptance of this limited definition was no doubt partly due to the advantage taken by some workers

of the property of swarming in isolating their strains from mixed cultures (Wenner and Rettger, 1919; Taylor, 1928), since these strains would also produce hydrogen sulphide and liquefy gelatin. *Proteus* organisms were broadly divided by maltose fermentation into *vulgaris* and *mirabilis* strains (Wenner and Rettger) and it was not until 1936 that the definition of the group was extended to include the non-gelatin-liquefying species of *P. morgani* (Rauss). Topley and Wilson (1936) used the property of gelatin liquefaction as a basis for classification, though St John-Brooks and Rhodes (1940) still relied on maltose fermentation and indole production. The increased study of urease production has led some American workers to regard this property as a more fundamental characteristic of *Proteus* organisms than gelatin liquefaction and certain other biochemical reactions; on this basis Rustigian and Stuart (1943, 1945) include *P. rettgeri* in the group in spite of mannitol fermentation and inability to produce hydrogen sulphide.

The majority of freshly isolated *vulgaris*, *mirabilis* and *rettgeri* strains can be readily distinguished by well defined and consistent biochemical reactions and there would seem to be considerable justification for according them specific names, as is already done with *P. morgani*. A knowledge of the characteristic biochemical reactions of the *Proteus* group is of value to the routine bacteriologist and a broad classification by which members of the group can be readily identified is suggested in the accompanying figure.

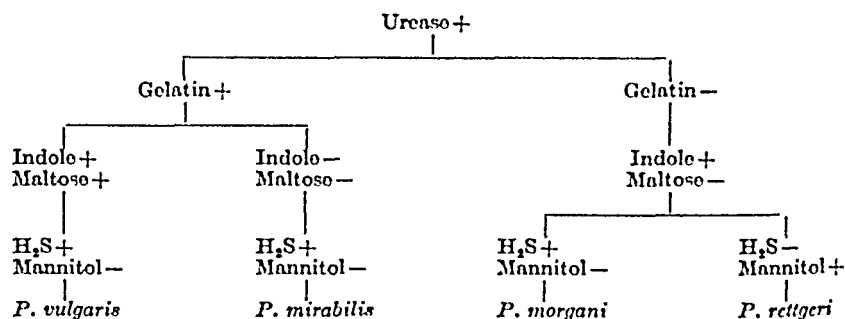


FIG.—A tentative classification of the *Proteus* group.

Morgan (1906, 1907) described a small number of strains which differ from his no. 1 bacillus in their failure to produce indole or in some other characteristic. In the absence of a fuller knowledge of the biochemical and serological behaviour of these organisms, it is doubtful whether they should still be included in the *Proteus* group. Kauffmann and Perch (1947), in a recent analysis of the antigenic structure of 538 *Proteus* organisms, confirmed that X19 and X2 strains resemble *P. vulgaris* in their biochemical reactions and XK strains resemble *P. mirabilis*. Although this work is not yet completed, the results already published indicate that the members of any one serological group have similar biochemical reactions.

Although the number of strains examined in this investigation is small, the results give some indication of the relative frequency of the different groups of *Proteus* organisms in faeces specimens obtained over a period of variable seasonable conditions from patients of all ages, the majority suffering from gastro-enteritis. A search of the literature has not revealed any analysis of comparable material as collections of strains previously submitted to detailed biochemical investigation have been derived from a variety of sources and frequently include a number selected from stock cultures. It is generally accepted that the great majority of gelatin-liquefying strains isolated from human sources fall into the *mirabilis* group. Levine (1942) found this figure as high as 90 per cent. of an unspecified number tested, and Rustigian and Stuart (1945) recorded 85.7 per cent. *mirabilis* strains out of 190 freshly isolated *vulgaris* and *mirabilis* cultures. In this investigation 14 per cent. of the 100 gelatin-liquefying strains were *P. vulgaris* and 86 per cent. were *P. mirabilis*.

#### SUMMARY

A study has been made of the urease and other biochemical reactions of 120 *Proteus* strains isolated from human faeces in a routine laboratory.

All strains showed a positive reaction on both strongly and weakly buffered urea media, though on the strongly buffered medium this reaction was considerably delayed with one strain. Rapid results were obtained with the weakly buffered medium and the value of this reaction in the routine investigation of non-lactose-fermenting organisms isolated from faeces is discussed.

Sixty-five paracolon bacilli were also tested for evidence of urease production. All were negative on the strongly buffered medium, but 8 strains, all citrate-positive, gave a delayed reaction on the weakly buffered medium. These results confirm the findings of previous workers.

As a result of the biochemical reactions all strains could be placed in 4 well defined groups: *P. vulgaris*, 14 strains; *P. mirabilis*, 86 strains; *P. morgani*, 9 strains; and *P. rettgeri*, 11 strains.

The biochemical behaviour and swarming properties of *P. rettgeri* have been investigated and the findings of recent workers confirmed. Evidence is produced in favour of including these strains in the *Proteus* group and a tentative classification is suggested.

My thanks are due to Mr O. Arthur for valuable technical assistance.

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## APPENDIX

MEDIA A AND B FOR TESTING THE UREASE REACTION OF  
PROTEUS

## Medium A

Yeast extract (Difco)	. . . . .	0.1 g.
Potassium dihydrogen phosphate ( $\text{KH}_2\text{PO}_4$ )	. . . . .	9.1 g.
Sodium phosphate ( $\text{Na}_2\text{HPO}_4$ )	. . . . .	9.5 g.
Agar	. . . . .	20 g.
Distilled water	. . . . .	1000 ml.

Steam until the solids are dissolved, filter through lint and add about 3 ml. of 0.4 per cent. phenol-red solution. Tube in 4.5 ml. quantities and autoclave at 10 lb. for 10 minutes. A 20 per cent. solution of urea is sterilised by Seitz filtration and 0.5 ml. added to each tube after cooling to 50° C. to make up a final medium with a concentration of 2 per cent. urea. The pH of the medium is 6.8-7.0.

## Medium B

Difco bacto peptone . . . . .	1 g.
Sodium chloride . . . . .	5 g.
Potassium dihydrogen phosphate ( $\text{KH}_2\text{PO}_4$ ) . . . . .	2 g.
Agar . . . . .	20 g.
Distilled water . . . . .	1000 ml.

Steam until the solids are dissolved, filter through lint and add 1 g. of glucose and about 3 ml. of 0.4 per cent. phenol-red solution. Adjust *pH* to 6.8-7.0, tube in 4.5 ml. quantities and autoclave at 10 lb. for 15 minutes. A 20 per cent. solution of urea is sterilised by Seitz filtration and 0.5 ml. added to each tube after cooling to 50° C. to make up a final medium with a concentration of 2 per cent. urea.

*Note.* Both media were tested for sterility by incubation at 37° C. for 24 hours.



## RAPID IDENTIFICATION OF PROTEUS

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In routine medical bacteriology organisms of the *Proteus* group are often encountered during the examination of pus, urine or faeces. With cultures of pus and urine most workers are content to rely on smell, swarming and gelatin liquefaction for routine diagnosis ; more elaborate biochemical investigations are rarely carried out. In the faeces, organisms of the *Proteus* group have considerable nuisance value because they may appear as discrete non-lactose-fermenting colonies, their swarming inhibited by the presence of bile salts in the medium.

Thus Hynes (1942) in a series of 353 faecal specimens found 10 per cent. of *Proteus* on direct plating on desoxycholate agar, whereas with tetrathionate broth 25 per cent. of the specimens examined yielded positive results. Rustigian and Stuart (1945) examined 110 specimens of faeces from normal people to determine the relative frequency of *Proteus*. On direct plating on eosin-methylene blue and SS agar 10 per cent. of the specimens yielded *Proteus*. After preliminary enrichment 36.3 per cent. were positive. *Proteus* is therefore a common organism in normal material, and although an experienced observer may suspect the nature of its pale colonies on faecal plates, they cannot be identified with certainty unless a series of reactions is carried out. Several colonies may have to be investigated, and this is both time-consuming and wasteful of material. A simple and rapid test for the identification and elimination of organisms of the *Proteus* group should therefore prove a useful adjunct to diagnostic technique.

For a single property to be of use in determining generic classification or as a screen test it must be a constant characteristic of the group not possessed by any organism from which the particular group has to be differentiated in practice. For the *Proteus* group such a property is found in the ability to break down urea. Urease activity of *Proteus* has been investigated by several workers. Moltke (1927) found that 194 strains of maltose- and non-maltose-fermenting organisms of the *Proteus* group all attacked urea. Rustigian and Stuart (1941) reported that all members of the genus except *Proteus hydrophilus*, *Pr. ichthyosmii* and *Pr. bombycis* decomposed urea with formation of sufficient ammonia to produce alkalinity detectable by means of an indicator. In 1942-43 Ferguson and Hook, examining 30 strains of *Proteus* from animal and human sources, used Rustigian and Stuart's, Ashworth's (1942) and their own method to detect urease activity. All but one species, *Pr. ichthyosmii*, gave positive results with all three tests.

This evidence shows that urease activity is a highly characteristic property of the *Proteus* group. If the reaction is to be used as a screen test for faecal pathogens it must, however, fulfil two further conditions. The first requirement

is that all the known pathogens must show an absence of urease activity. For this purpose it is clearly not enough to accept a small group as representative if false generalisations are to be avoided; for example one might thus be led into the fallacious statement that all *Salmonella* strains are indole negative or non-liquefiers of gelatin. Ferguson and Hook tested 75 *Salmonella* strains for urease activity and found them all negative.

Although not essential for the purposes of the screen test, it is desirable that other non-lactose-fermenting Gram-negative bacilli should also give negative results. Ashworth tested 30 strains of paracolon bacilli and found them all non-urease producers. Schneider and Gunderson (1946) found one strain of *Bacterium aerogenes* and a strain of *Brucella abortus* urease positive by their method. Christensen (1946) reported that with his method 21 of 44 paracolon strains examined gave positive results in times ranging from 6 hours to 6 days. He concluded that paracolon *Aerobacter* (= *aerogenes*) and paracolon intermediates are urease positive, but that paracolon *Escherichia* (= *coli*) are negative.

The second requirement is that the test should be easy and rapid to perform, since a time-consuming test is useless for screening purposes. Most urease tests now in use require at least 24 hours' incubation, during which time the organism can with little more trouble be put through the classical tests. Stuart, van Stratum and Rustigian (1945) described a rapid method, but it required large inocula. Schneider and Gunderson as well as Christensen described methods giving quick results, but none of these was designed for the investigation of single colonies. This paper describes my attempt to work out a test which would agree in its results with the established urease methods and could be completed in a few hours.

## TECHNICAL METHODS

### *Tests for urease activity*

Three methods were employed for the detection of urease activity. The first of these was the procedure used by Rustigian and Stuart, the second a modification of it; the third was a method designed to give rapid results.

*Method 1* (Rustigian and Stuart). The original medium consisted of 2 per cent. urea (Merck), 0.01 per cent. yeast extract (Difco) and *M/15* primary and secondary phosphate buffers (Sorensen) in distilled water, to yield a pH of 6.8. Because of difficulties of supply, Analar urea and marmite were substituted for the Merck and Difco products originally specified. This medium is sterilised by Seitz filtration and distributed in 5-ml. amounts in sterile tubes. The test organism is inoculated heavily into the medium from a 24-hour agar-slope culture and the tube incubated for 24 hours at 37° C. Thereafter two drops of brom-thymol-blue indicator are added. Tubes in which urea has been broken down with formation of ammonia develop a blue colour; a negative reaction is indicated by a pale green colour.

*Method 2* is a modification of the previous method, nesslerisation replacing the use of an indicator. After 24 hours' incubation 0.5 ml. of the culture is transferred to a clean Kahn tube and 0.1 ml. of Nessler's reagent is added to it. A positive reaction is indicated by the formation of a brown precipitate, negative tubes remaining colourless. A blank control and a test with a known non-urease-producing organism are included in each batch.

*Method 3* is the rapid test designed for use in the identification of the *Proteus* group. The substrate consists of 2 per cent. urea in Clark and Lubs's phosphate buffer at pH 7.2 and is prepared as follows. To 50 ml. of 0.2 *M* acid potassium phosphate add 35 ml. of 0.2 *N* NaOH and 4 g. of pure urea and make up the volume to 200 ml. with ammonia-free distilled water.

Sterilisation of this substrate is not required. It should be kept in a bottle

with a vaseline-smear glass stopper and stored in the refrigerator when not in use. In this way it keeps for about a month. Freshly prepared substrate should be tested with a known urease-producer, but for the actual test only a negative control and an uninoculated blank need be included. The tubes are not sterilised, but the glassware must be scrupulously clean.

To carry out the test a convenient amount of growth is removed with a cold wire from a 24-hour nutrient agar slope and emulsified in 0.5 ml. of the substrate in a Kahn tube. The inoculum should be heavy enough to give a slight but distinct opalescence to the fluid viewed against a dark background. The Kahn tube is now placed in a water-bath at 37° C. for exactly 3 hours. At the end of that time it is removed and 0.1 ml. of Nessler's reagent is added. Readings are taken 3 minutes after the addition of the Nessler reagent. Both the blank and the negative control should be absolutely colourless. A positive reaction is shown by a yellow colour which ranges from a pale but distinct yellow to a dark-brown precipitate. The strength of the reaction must not, however, be taken as indicative of the vigour with which urea is attacked, for organisms from the same culture may yield both "weak" and "strong" reactions on repeated testing, since the concentration of ammonia formed from urea under these conditions does not show a linear increase. In fact quantitative tests at 3-minute intervals give an ammonia-concentration curve with spikes at intervals of about 20 minutes. There is a rapid increase in ammonia, followed by an equally rapid decrease, representing utilisation of ammonia. The increase is always greater than the utilisation and in this way ammonia accumulates. It was found by experiment that 3 hours at 37° C. is the minimum time of incubation for reliable results. The strength of the reaction is variable because at the time of nesslerisation the culture may be in the ammonia-production stage (strong reaction) or ammonia-utilisation stage (weak reaction). The incubation should be for precisely 3 hours, since at this time most of the cultures seem to give a strong reaction.

In using method 3 as a screen for isolated non-lactose-fermenting colonies a slight modification was introduced. To allow for the smaller inoculum the volume of the substrate was reduced to 0.3 ml. and one drop of Nessler's reagent was used in place of 0.1 ml. Readings were taken 4-5 minutes after nesslerisation.

The method devised by Ferguson and Hook was also tried, but it gave disappointing results in my hands.

### *Comparison with other techniques*

Stuart, van Stratum and Rustigian claimed that their rapid method gave positive results in times ranging from 40 seconds to 80 minutes with large inocula of the order of 3 loopfuls from agar slopes. It appeared desirable therefore to determine by titration the minimum amount of urea destruction necessary to produce a positive reaction. It was found that hydrolysis of 0.12 mg. of urea was necessary to produce the required colour change in the medium of Stuart *et al.*, whereas 0.012 mg. was sufficient to give a definite yellow colour with nesslerisation in the medium described for method 3 in this paper.

Stuart *et al.* also stressed that in strongly buffered urea medium only members of the genus *Proteus* give evidence of urea utilisation, whereas in weakly buffered medium coliform and paracolon bacilli may also give the reaction. To obtain maximum sensitivity for their rapid test, which is a modification of the Rustigian and Stuart method

described under method 1, they had to reduce the buffer to a point just sufficient to maintain the reaction of pH 6.8 in ordinary glassware. Thus the medium used for their rapid test has a molar concentration of 0.0013 *M* as against the medium described for method 3 of this paper, which has a concentration of 0.05 *M* and appears to be about ten times more sensitive and possesses the advantage of a high molar concentration of the buffer. The relative sensitivity of the two tests was further illustrated when 35 *Proteus* strains were tested by both methods in parallel with identical inocula of about 500,000 organisms. All were positive with method 3, whereas none was positive with the rapid method of Stuart *et al.* in 3 hours.

Schneider and Gunderson described a complicated method for which they claimed that positive results were usually discernible after 6-8 hours' and were very prominent after 18-24 hours' incubation at 37° C. Their medium contained peptone, which is an alternative source of nitrogen, and they stressed the need for a large inoculum. Another modification in which urea was not the sole source of nitrogen was described by Christensen. Heavy inocula were necessary and the earliest recorded results were obtained after 6 hours' incubation. These two methods appear to offer few advantages; they are relatively slow, require large inocula and contain an alternative source of ammonia which may be the cause of their apparently lesser selectivity.

#### TESTS FOR OTHER PROPERTIES

Three other properties commonly regarded in routine work as characteristic of the *Proteus* group were also investigated. These were smell, swarming and gelatin liquefaction. For the detection of smell the organisms were grown on nutrient-agar slopes for 24 hours at 37° C. in screw-capped bottles and smelled immediately after removal from the incubator. Swarming was tested for by using decreasing concentrations of agar. Since commercial agar preparations show considerable differences, percentages are misleading. The agar used was such that the lowest concentration with which a slope could be made was 1.5 per cent.; plates had to be used for 1 per cent. agar. Every strain was first tested on 2.5 per cent. agar for 24 hours at 37° C. If it failed to swarm on this it was re-tested on 2 and 1.5 per cent. agar in succession until swarming was observed. Strains which failed to spread were re-tested on 1 per cent. agar plates at 21° C. for 24 hours and a second reading was taken after 48 hours. Gelatin liquefaction was tested for in the usual way, readings being taken after growth for 48 hours at 21° C.

#### RESULTS

All these tests were applied to a series of 125 strains of organisms considered to be members of the *Proteus* group. Of these 82 were obtained from the National Collection of Type Cultures and 43 were

recently isolated from human sources. In addition 176 strains of *Salmonella* and 72 strains of *Shigella* were subjected to the three tests of urease activity and 63 further strains of *Shigella*, 76 miscellaneous strains of paracolon bacilli, 20 strains of coliform bacilli (including 9 late-lactose-fermenting strains appearing as pale colonies on MacConkey's medium) and 8 strains of *Pseudomonas pyocyanea* were tested by the rapid test only. A single strain of *Brucella melitensis* was also tested.

The findings with the 125 strains of *Proteus* are set out in the table (p. 188). Identical results were obtained with methods 1, 2 and 3. Ten of the 125 strains were found to be non-urease producers. As regards the other properties of the group, 4.8 per cent. of the organisms exhibited no appreciable smell, but by no means all the remainder had the smell one associates with *Proteus*. Cultures on all the media employed failed to elicit swarming in 20.8 per cent. of strains and only 54.6 per cent. swarmed on agar of ordinary strength (2.5 per cent.). Incubation at 21° C. on 1 per cent. agar was required to elicit the property in 22 strains (22.6 per cent. of the swarming strains). Gelatin was liquefied within 48 hours by 49 strains only (39.2 per cent.).

The tests for urease activity carried out on 176 *Salmonella* strains, 135 *Shigella* strains, 76 paracolon strains, 20 strains of coliform bacilli and 8 strains of *Ps. pyocyanea* all yielded negative results. A list of the names of all *Salmonella*, *Shigella* and paracolon strains tested has been lodged with the Librarian, General Library, British Museum (Natural History), London, S.W. 7.

As a further assessment of the 3-hour test under working conditions 25 strains of *Salmonella* and 25 of *Proteus* were plated on MacConkey's and Leifson's media. Single colonies from the resulting growths were then put through the rapid test. Of 50 *Proteus* colonies thus tested 46 yielded clear-cut positive results while 3 gave weakly positive results and there was one false negative. This last test was carried out with a very small colony. The *Salmonella* strains gave consistently negative results from both media.

#### DISCUSSION

Urease production proved to be the most constant property of the *Proteus* group. Of the 125 strains tested ten only were non-urease producers. Rustigian and Stuart obtained the same results with two of these, *Pr. bombycis* and *Pr. hydrophilus*, and suggested their exclusion from the genus *Proteus*, while St John-Bropks and Rhodes (1940) made the same suggestion about *Pr. hydrophilus*, but on different grounds. On the other hand Ferguson and Hook found two strains of *Pr. hydrophilus* positive to the urease test, but Kulp and Borden (1942), in an extensive study of this organism, found that it was a non-urease producer. Strains 29, 30 and 31 in the table



TABLE—Characters of *Proteus* strains examined

Serial no.	Strain		Characters			
	Name	Official no.*	Small	Swarming	Gelatin liquefaction	Urease
1	<i>americanus</i>	789 B 1	+	+	+	+
2	"	(N.C.T.C. 6255a)	+	+	+	+
3	"	789 B 2	+	+	+	+
4	<i>ammoniac</i>	(N.C.T.C. 6255b)	+	—	—	+
5	"	A.T.C.C. 4676 ;	+	+	+	+
6	<i>anindologenes</i>	N.C.T.C. 5872 ;	+	+	+	+
7	"	A.T.C.C. 7002 ;	+	+	+	+
8	"	N.C.T.C. 5873	+	+	+	+
9	"	N.C.T.C. 59	+	+	+	+
10	"	" 60	+	+	+	+
11	"	" 1632	+	+	+	+
12	"	" 3177	+	+	+	+
13	<i>bombycis</i>	" 5039	+	+	+	+
14	<i>hydrophilus</i>	" 5586	+	+	+	+
15	<i>vulgaris</i>	" 5821	+	+	+	+
16	"	" 2896	+	+	+	+
17	"	A.T.C.C. 887 ;	—	—	—	—
18	"	N.C.T.C. 5874	+	—	—	—
19	"	" 5903	+	—	+	—
20	"	" 401	+	—	+	—
21	"	" 402	+	+	+	+
22	"	" 3156	+	+	+	+
23	"	" 4109	+	+	+	+
24	"	" 4445	+	+	+	+
25	"	" 5887	+	+	+	+
26	"	" 6789	+	—	—	+
27	"	" 6790	+	—	—	+
28	<i>para-americanus</i>	" 6792	—	+	+	+
29	<i>sp. anaerogenic</i>	" 5227	+	+	+	+
30	" Kohn	" 6369	+	+	+	+
31	<i>rettgeri</i>	" 5857	+	+	+	+
32	<i>sp.</i>	" 1501	+	+	+	+
33	" Elmes	" 6300	+	—	+	+
34	"	" 6706	+	+	+	+
35	"	" 6952	+	—	—	—
36	"	" 6953	+	—	—	—
37	" Haines	" 6197	+	+	+	+
38	"	" 5431	—	+	—	+
39	"	" 5130	+	+	—	—
40	<i>mirabilis</i>	" 5132	+	+	—	—
41	<i>pseudovaleriae</i>	" 4175	+	+	—	—
42	<i>vulgaris</i>	" 2518	+	+	—	+
43	"	" 6791	+	—	—	—
44	X strain XK (H)	" 2100	+	+	+	+
45	" XK (O)	" 2091	+	+	+	+
46	" XL (H)	" 2091a	+	—	—	+
47	" XL (O)	" 4636	+	—	—	+
48	" X2 (H)	" 4635	+	—	+	+
49	" X2 (O)	" 3307	+	+	+	+
50	" X19 (H)	" 3307a	+	—	—	+
51	" X19	" 3137	+	+	—	+
52	" OX19	" 7052	+	—	—	+
53	" OX19 (H)	" 67	+	+	+	+
54	" OX19	" 1508	+	+	—	+
55	" OX19	" 1620	+	+	+	+
56	" OX19 (O)	" 2462	+	+	+	+
57	" OX19	" 3138	+	+	+	+
58	" OX19	" 7050	+	—	+	+
59	" OX19 (O)	" 7051	+	+	—	+
60	" OX19	" 1178	+	+	+	+
61	<i>morganii</i>	" 2475	+	+	—	+
62	"	" 234	+	—	+	+
63	" 692	" 235	+	+	+	+
64	"	" 417	+	+	+	+
65	"	" 679	+	—	+	+
66	"	" 1707	+	—	+	+
67	"	" 1708	+	—	+	+

\* N.C.T.C. = National Collection of Type Cultures.  
A.T.C.C. = American Type Culture Collection.

TABLE—Characters of *Proteus* strains examined—continued

Serial no.	Strain		Official nos.*	Characters			
	Name			Smell	Swarm- ing	Gelatin lique- faction	Urease
63	<i>morgani</i>		N.C.T.C. 1709	+	—	—	+
64	"		2814b	+	—	—	+
65	"		2818	+	+	+	+
66	" 299		3389	+	+	—	+
67	" 505		6794	+	+	—	+
68	" 506		6793	+	+	—	+
69	"		6795	+	—	—	+
70	"		232	+	+	—	+
71	"		2814	+	+	—	+
72	"		2815	+	+	—	+
73	"		2817	+	—	—	+
74	" Thjotta		3518	+	—	—	+
75	<i>morgani</i> , Castellani and Douglas, group A		3663	—	+	—	+
76	<i>morgani</i> , Castellani and Douglas, group C		3665	+	—	—	+
77	<i>morgani</i> , O form		3822	+	+	—	+
78	<i>morgani</i> , Castellani and Douglas, group D		3666	+	+	—	+
79	<i>morgani</i> , Castellani and Douglas, group E		3667	+	+	—	+
80	<i>morgani</i>		2814a	+	+	—	+
81	" 6		5845	+	+	—	+
82	"		3069	+	+	—	+
83	<i>vulgaris</i> .		L 26	+	+	—	+
84	<i>morgani</i>		L 30	+	+	—	+
85	<i>vulgaris</i>		L 35	+	+	—	+
86	"		S.G.H. 4857	+	+	—	+
87	"		L 45	+	+	—	+
88	"		L 62	+	+	—	+
89	"		L 66	+	+	—	+
90	"		L 68	+	+	—	+
91	<i>morgani</i>		L 72	+	+	—	+
92	"		L 73	+	+	—	+
93	"		L 78	+	+	—	+
94	"		L 80	+	+	—	+
95	<i>vulgaris</i>		L 85	+	+	—	+
96	<i>morgani</i>		L 84	+	+	—	+
97	"		L 87	+	+	—	+
98	"		L 97	+	+	—	+
99	"		L 114	+	+	—	+
100	"		L 116	+	+	—	+
101	"		L 120	+	+	—	+
102	"		L 121	+	+	—	+
103	"		L 122	+	+	—	+
104	"		L 130	+	+	—	+
105	"		L 166	+	+	—	+
106	"		L 176	+	+	—	+
107	<i>vulgaris</i>		L 185	+	+	—	+
108	<i>morgani</i>		L.P.M.	+	+	—	+
109	"		S.G.H.	+	+	—	+
110	<i>vulgaris</i>		"	4809	+	+	+
111	<i>morgani</i>		"	2304	+	+	+
112	<i>vulgaris</i>		"	4969	+	+	+
113	<i>morgani</i>		"	4984	+	+	+
114	<i>vulgaris</i>		"	2854	+	+	+
115	"		"	R 1	+	+	+
116	"		"	R 2	+	+	+
117	<i>morgani</i>		"	1790	+	+	+
118	"		"	2167	+	+	+
119	<i>vulgaris</i>		"	"	+	+	+
120	<i>morgani</i>		"	3178	+	+	+
121	<i>vulgaris</i>		"	5699	+	+	+
122	"		"	4712	+	+	+
123	<i>morgani</i>		"	L 128	+	+	+
124	<i>vulgaris</i>		"	"	+	+	+
125	"		"	"	+	+	+

were, according to information given by the National Collection of Type Cultures, isolated by Dr B. G. T. Elmes in Nigeria during 1944-45 from cases of dysentery. Organisms of the same type were isolated from 26 cases in the course of 2 years. The three strains gave identical reactions; they were motile Gram-negative bacilli, producing acid in 24 hours in glucose, maltose, mannitol and sorbitol. Lactose, sucrose and dulcitol were not fermented. The indole reaction was negative and litmus milk showed no change. Serological tests excluded them from the *Salmonella* group. They were tentatively regarded as *Proteus* strains and included in the National Collection. As is seen from the table, they show neither swarming, urease production nor gelatin liquefaction. Strains 33-35 are 3 of 7 strains isolated by Haines (1938) from rotten eggs. On isolation they showed liquefaction of gelatin, which is recorded as "slow" (time not stated). One of the strains showed late lactose fermentation. They were tested for urease activity on isolation and all found negative. On account of certain atypical sugar fermentations they were regarded as new species of *Proteus*. Strain no. 37, *Proteus pseudovaleria*, was isolated by de Assis (1927-28) by blood culture from a man with a typhoid-like disease. Its inclusion in the genus is dubious. It is a Gram-negative motile bacillus fermenting mannitol and salicin but not sucrose and showing late lactose fermentation. The organism neither swarms nor liquefies gelatin and does not appear to have been tested for urease when first isolated. Strain no. 74, *Pr. morgani* of Thjøtta, was an old laboratory strain and no information is available about it.

Whether urease production is an absolute generic characteristic is largely a matter of taxonomy. If we agree with Moltke, Rustigian and Stuart, and Topley and Wilson (1946), and exclude non-urease producers from the genus, then the test is clearly most suitable for the rapid identification of the group. Of the Gram-negative bacilli tested, only *Br. melitensis* gave a positive reaction in 3 hours with method 3. But since colonies of the *Brucella* group will not be encountered on the media used for the routine culture of faecal material, this will hardly lead to an error in the screen test.

Even if one does not accept urease production as an absolute generic characteristic, the large majority of strains (92 per cent.) and certainly all the common and recognised types appear to be identifiable by means of this single criterion. None of the other properties so commonly used for identification of the *Proteus* group showed equal reliability as a criterion for group identification. The negative results obtained with strains of *Salmonella*, *Shigella*, coliform bacilli, *Ps. pyocyanea* and paracolon bacilli indicate that the urease test is suitable for use as a screen test in the examination of faeces. To carry out the test the non-lactose-fermenting colony under investigation is lightly touched with a wire and inoculated into peptone water which is incubated and used for further tests if the urease reaction is negative.

The entire remainder of the colony is then used for the rapid test as described in method 3.

The available evidence suggests that urease is a constitutive enzyme of the genus *Proteus*. The method here described is essentially a microchemical method for the detection of a constitutive enzyme and will yield positive results whether the culture medium is nutrient agar, MacConkey's or Leifson's medium. Methods involving the use of media containing an alternative source of nitrogen are subject to the criticism that alkaline end-products may originate from sources other than urea and may produce a colour change in the indicator. Prolonged incubation on the other hand may bring adaptive enzymes into play. The rapid nesslerisation method (method 3 of this paper) cannot be criticised on these grounds, and on account of its specificity for ammonia and its great sensitivity satisfactory results can be obtained with single colonies within 3 hours, an advantage not shared by any of the methods so far described.

### SUMMARY

1. A simple and rapid micro-chemical test is described for the detection of urease production by bacteria in young cultures.

2. Urease production is a highly characteristic feature of organisms of the *Proteus* group. Of 125 strains tested 115 (92 per cent.) gave positive results. The possibility is discussed that the negative reactors were erroneously assigned to the group.

3. Negative reactions were obtained with 176 strains of *Salmonella*, 135 strains of *Shigella*, 20 strains of coliform bacilli, 76 paracolon strains and 8 strains of *Ps. pyocyanea*.

4. The test, which can be carried out in 3 hours, should be of use in the recognition of the *Proteus* group and as a screen test in the bacteriological examination of faeces.

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ADAMANTINOMA OF THE JAW WITH  
PULMONARY METASTASES

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(PLATES XXX AND XXXI)

ADAMANTINOMA of the jaw is rarely accompanied by metastases and is therefore as a rule treated conservatively. The case here reported shows some unusual features and it is suggested that the excision of such tumours should be more radical than the operation generally carried out.

## CASE REPORT

*Clinical history*

The patient, a sheep-farmer aged 57, was admitted to Napier Hospital in April 1943 complaining of a swelling of the lower jaw first noticed 10 days before; he mentioned that his lower denture had been fitting badly for 6 months. Radiography showed a punched-out rarefaction in the region of the symphysis menti. This was thought to be due to a dentigerous cyst. The lesion was curetted and the material sent to a pathologist, who reported it as "poorly-differentiated squamous-cell carcinoma". X-ray therapy was administered to both submaxillary regions.

Fifteen months later (July 1944) the patient was re-admitted to hospital with a firm swelling about the size of a walnut in the floor of the mouth. There had been a gradual recurrence at the site of the original tumour. No enlarged lymph-nodes were palpable. He complained of being "short of breath on the hills", but apparently no X-ray examination of the chest was carried out. The tumour was excised locally and was shown histologically to be an adamantinoma.

In May 1945 he was again admitted with complaint of shortness of breath, tiredness and slight cough of about 6 months' duration. Radiography of the chest showed numerous sharply defined rounded opacities of various sizes in both lungs (fig. 1). These were regarded as secondary tumours or possibly hydatid cysts. The Casoni skin test for echinococcal infestation was negative. A trial dose of deep X-rays (2400 r) was given to a portion of the thorax, but a film taken one week later showed no appreciable effect on the size or density of the opacities.

The patient was first seen by one of us (G. E. W.) in October 1945, when his chief complaint was of tiredness and shortness of breath on slight exertion. He had a slight cough with a small amount of sputum. He was a tall, rather thin man weighing 10 stones. Colour and appearance healthy, except that he appeared rather worn and frail for a farmer of his age (59). Pulse normal; blood pressure 120/80. He was edentulous, and no tumour of the jaw was

noticed at this time. There was no deformity of the thorax and the heart was clinically normal. Relative dullness was found at the base of each lung, especially the right, with much diminution of air entry.

He was kept under observation, and in December 1945 he complained of another recurrence of the tumour of his jaw. A firm bluish epulis about the size of a walnut was found in the region of the symphysis menti; it was painless but caused difficulty in mastication by interference with his denture. X-ray examination of the lower jaw showed a large bony defect (fig. 2).

At operation on 9th January 1946, under pentothal anaesthesia, the mass was found to be partly cystic, with soft bony trabeculae in the solid parts. The growth was removed, mainly by curettage, and sent for examination. The patient recovered rapidly from this operation, but 3 weeks later his condition began to show obvious deterioration; the sputum became more copious and he showed great weakness and loss of weight. The chest was again radiographed in March 1946, when a visiting radiologist of great experience, on reviewing the chest-films, expressed a strong opinion that the pulmonary "tumours" were hydatid cysts, and urged exploration. It was thought that an adamantinoma of the jaw would be very unlikely to give rise to multiple large pulmonary metastases. Accordingly on 21st March, under avertin, nitrous oxide and oxygen, thoracotomy was performed by an incision in the eighth left intercostal space. The presenting portion of the lung was found to consist of greyish solid tumour tissue, not adherent to the parietal pleura, and a small portion was removed for examination.

The patient recovered from the thoracotomy operation, but the next day he sank rapidly into unconsciousness and died. Permission for an autopsy was not obtained.

### *Pathology*

The sections from the original tumour of the jaw (April 1943) were fortunately available; these showed some resemblance to squamous carcinoma, but differed little in histological structure from the later material, and we have no doubt that the original tumour was an adamantinoma.

The microscopic appearance of the first recurrence (July 1944) was similar to that shown in fig. 3. The tumour shows no cystic structure, but is composed of convoluted columns of epithelial cells, between which lie strands of moderately vascular connective-tissue stroma. There are also numerous irregular islets of an acidophilic hyaline or slightly granular substance, which probably represents enamel pulp. The tumour cells are fairly uniform in size, and at the periphery of the columns they tend to be columnar. Typical columnar ameloblasts are not present, but some of the central cells of the tumour columns show the stellate appearance of the cells of the enamel-organ. There are very few mitoses, and the usual signs of anaplasia and active malignancy are absent. The histological picture seems to agree with that of the adamantinoma classed by Ewing (1934) as "plexiform epithelioma".

The microscopic appearance (fig. 3) of the second recurrence (January 1946) is similar to that of the first. The cells are still well differentiated and show no evidence of a change towards a more anaplastic type. Yet this material was obtained some 8 months after

ADAMANTINOMA WITH PULMONARY METASTASES

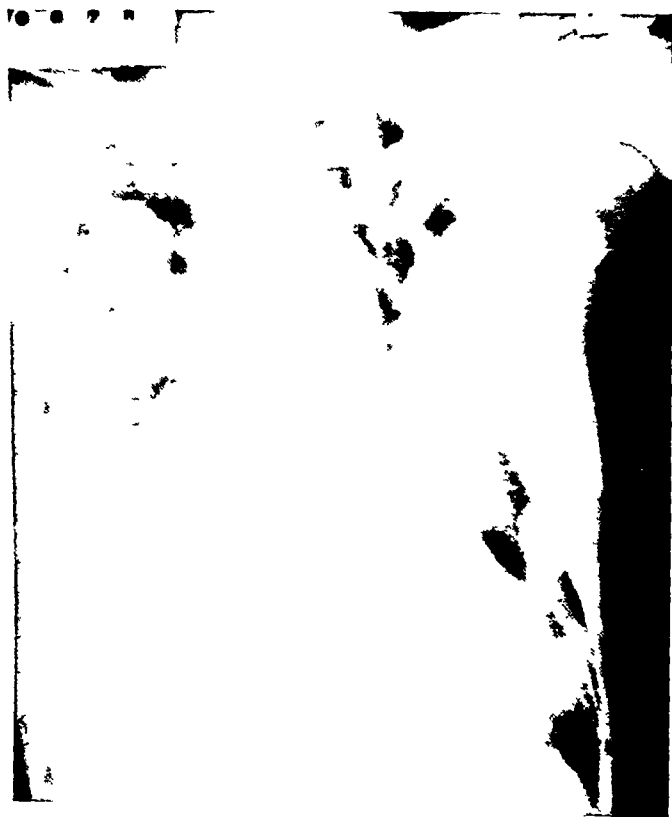


FIG. 1.—Radiograph of chest, May 1945, showing multiple rounded opacities in both lung fields.



FIG. 2 —Radiograph of lower jaw, December 1945





the radiological discovery of the pulmonary opacities and 18 months after the first complaint of dyspnoea.

The tissue from the lung, however, shows a distinctly more active growth (fig. 4), although successive radiographs of the chest showed only a slow increase in the size of the shadows. The tumour here is undoubtedly of the same type as and in places appears almost identical with the primary growth in the jaw, but there is a tendency to the formation of larger cell masses and mitoses are more numerous.

### DISCUSSION

It is generally recognised that adamantinoma of the jaw has a pronounced tendency to local recurrence and extension, but metastases are apparently rare. In a review of 196 cases McFarland and Patterson (1931) accept only two as showing proof of metastases, while Ivy and Curtis (1937) go so far as to say that "metastases are almost unknown". Nevertheless there are at least seven previous reports of presumed pulmonary metastases from primary adamantinoma of the jaw; most of these reports are incomplete, and only Vorzimer and Perla (1932) provide histological confirmation of the nature of the pulmonary lesions. There are several recorded cases of histologically proved metastases in the cervical lymph-nodes, but these we do not propose to discuss here.

#### *Summary of previously reported cases of pulmonary metastases*

**Cases 1 and 2 (Ewing, 1934, p. 758).** "In one very extensive fibro-epithelial tumour I found metastases in one cervical node and a small nodule in the lung. In another very malignant case the fourth recurrence was in the cervical nodes and loose tissues of the neck and probably in the lungs".

**Case 3 (Weissenfels, 1922, quoted by Chont).** A histologically confirmed adamantinoma of the left mandible, recurring in the jaw; duration 14 years; clinical and radiological evidence of metastasis to the lung.

**Case 4 (Simmons, 1928).** In case 7 in his series "there was evidence of lung metastases" a month before death. The nature of the "evidence" is not mentioned. Metastases in the cervical lymph-nodes are stated to have been histologically confirmed. The patient had several operations on the jaw; death occurred 15 years after the first appearance of the tumour.

**Case 5 (Vorzimer and Perla, 1932).** Adamantinoma of maxilla with invasion of antrum; duration 21 years. At least seven operations for recurrence, and one radium needling. Extensive secondary tumour in lower lobe of right lung, confirmed by thoracotomy, bronchoscopy and autopsy, with microscopic examination of sections.

**Case 6 (Havens, 1939).** Case of adamantinoma in which a diagnosis of tumour of the lung was made at the time of death; there are neither histological details nor any mention of necropsy.

**Case 7 (Chont, 1943).** A man of 55; duration of tumour of jaw 11½ years before "extensive bilateral pulmonary metastases" were found; the diagnosis of pulmonary metastases was made radiologically. A punch-biopsy from the lung showed only necrotic material with occasional cellular fragments; there was therefore no satisfactory histological confirmation. Chont also quotes a case from Horsley (1924) as showing metastases in the lung, but reference to the original paper fails to confirm this.

In all the previously recorded cases where details are given, it is noteworthy that there was an interval of well over ten years between the first appearance of the adamantinoma of the jaw and the occurrence of pulmonary metastases. In the present case large tumours were shown by X-ray examination of the chest only 2½ years after the patient first noticed difficulty with his dental plate, which may be taken as indicating the onset of expansion of the mandible. It is obvious that metastasis to the lungs must have occurred much earlier than the date of the first X-ray examination of the chest. Yet the microscopic appearance of the tumour of the jaw did not suggest high malignancy, and one was reluctant to believe that this slow-growing, rather well differentiated adamantinoma could be the source of such large pulmonary secondaries.

Some of the previous reports of secondary adamantinoma in the lungs have referred to the dedifferentiated appearance of the tumour which, after many recurrences in the jaw, had shown transformation to a frankly carcinomatous or even sarcomatous structure.

Vorzimer and Perla, however, stress the well differentiated histological appearances in their material, and their photomicrographs (not unlike those reproduced here) suggest a relatively benign neoplasm or at least one of slow growth. Faced with the apparent paradox of a relatively benign primary growth producing large pulmonary metastases, Vorzimer and Perla put forward the suggestion that particles of the primary tumour may have been inhaled into the lung. This would seem a reasonable enough explanation in their case, in which the main bronchus to the right lower lobe was found to be filled with tumour-tissue, and in which the primary adamantinoma was situated in the upper jaw and had spread into the maxillary antrum and nasal fossa. Indeed, such an authority as Willis (1934) accepts this as probably almost the only reported case in which the spread of a tumour by aspiration seems likely. In our own case there was no ulcerating or fungating mass of growth, and aspiration of tumour tissue into the bronchi seems unlikely.

The other possible modes of spread are by the lymphatics and by the blood stream. Lymphatic permeation is improbable, as there was no clinical evidence of invasion of the cervical lymph-nodes, such as has been reported in a number of cases of adamantinoma of the jaw (Simmons, 1928; New, 1929; Gentsch, 1932; Havens, 1939). We are left, therefore, with the blood-borne route as the most likely mode of spread from jaw to lungs in this case. An additional point which is perhaps in favour of blood stream spread is the "cannon-ball" appearance of the pulmonary tumours (fig. 1).

In conclusion, it seems that an adamantinoma of the jaw, no matter how well differentiated and benign it appears histologically, may occasionally produce distant metastases. In view of this possibility, radical excision of the portion of bone involved should be the accepted method of surgical treatment in preference to simple curettage.

ADAMANTINOMA WITH PULMONARY METASTASES



FIG. 3.—Tumour of jaw : second recurrence.  $\times 125$ .

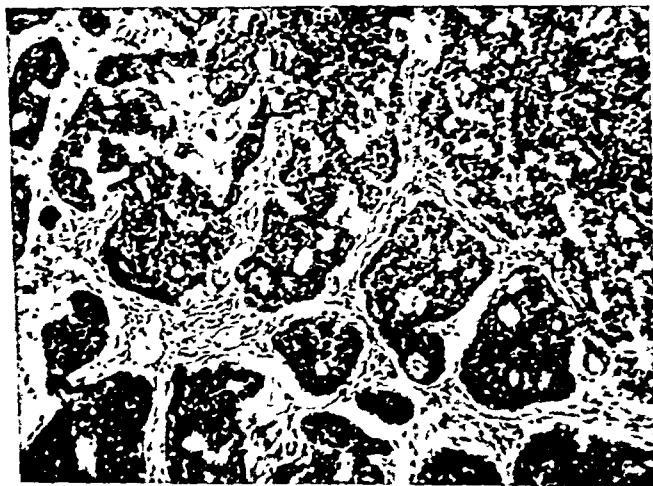


FIG. 4.—Secondary adamantinoma in lung. Note the formation of large solid cellular masses in the lower part of the field.  $\times 125$ .



## SUMMARY

A case of adamantinoma of the lower jaw is described, with extensive histologically confirmed pulmonary metastases.

In a review of the literature reports are found of seven possible cases of secondary pulmonary adamantinoma, one of which was histologically confirmed. The mode of spread in such cases is discussed, and a plea is made for more radical surgical treatment of adamantinoma.

Our thanks are due to Dr J. Foley (Medical Superintendent, Napier Hospital) for permission to make use of the hospital records, to Professor Alan C. Lendrum for his helpful criticism and to Miss M. Runnicles for assistance with the photomicrographs.

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576 . 8 . 097 . 5 : 576 . 851 . 214 (*Str. hæmolyticus*,  
*viridans* variants)

## ANTI-HÆMOLYTIC ACTION OF *VIRIDANS* ( $\alpha$ ) VARIANTS OF HÆMOLYTIC STREPTOCOCCI

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A USEFUL method of inducing *viridans* variants of hæmolytic streptococci consists in growing these under raised oxygen tension (Isaacs, 1947). The question arises as to the nature of the changes involved in this transformation. Todd (1928) investigated a non-hæmolytic variant obtained by passing a hæmolytic streptococcus through mice; hæmolysin production in shallow layers of serum-broth was inhibited if either the hæmolytic organism or a heterologous hæmolytic strain was grown along with the non-hæmolytic variant, but one strain of *Streptococcus viridans* did not act in this way. It was concluded that the variant strain produced a substance which destroyed its own hæmolysin as well as that of other hæmolytic streptococci grown along with it, but the anti-hæmolytic substance was not obtained in filtrates of cultures and could not be identified. The present report deals further with the anti-hæmolytic property of *viridans* variants.

### *Effect of $\alpha$ variants on hæmolysin production by hæmolytic streptococci when grown along with them*

Hæmolytic streptococci were incubated at 37° C. for six hours along with their  $\alpha$  variants (Isaacs) in shallow layers of 20 per cent. horse- or sheep-serum broth. The hæmolysin titres of the whole cultures, or of the supernatants after centrifuging, were estimated throughout with 0.5 c.c. of a 5 per cent. saline suspension of washed sheep red cells, the mixtures being kept at 37° C. for one hour, when the results were read. Further incubation overnight at 5° C. did not significantly alter the M.H.D. Under these conditions hæmolysin production was more or less completely inhibited. Plating of the mixed culture showed the presence of hæmolytic and *viridans* colonies in about equal numbers. Variant strains also inhibited hæmolysin production by heterologous  $\beta$  strains. Thus variants obtained from

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\* McCunn Research Scholar.





very labile at 37° C. When a mixture of equal parts of hæmolysin and serum broth was inoculated with a variant and tested after keeping for 3 and 6 hours at 37° C., it was not destroyed more rapidly than in a sterile control mixture of hæmolysin with fresh serum broth. Filtrates of variant cultures are actually less anti-hæmolytic than a control of uninoculated horse-serum broth, the slight inhibitory action of which is due to the serum. A mixture of streptolysin-S (Todd, 1938) with hydrogen peroxide to give a final concentration of 1/10 volume was allowed to stand for one hour at room temperature, after which it showed the same titre as a control with saline. Thus, in agreement with the results of Todd (1928) and Fuller and Maxted (1939), peroxide in the concentrations used does not inhibit the action of pre-formed hæmolysin.

*Effect of peroxide on the extraction by serum of hæmolysin  
from hæmolytic streptococci*

Weld (1934, 1935) extracted a powerful hæmotoxin from the deposit of a centrifuged broth culture of a hæmolytic streptococcus by shaking for one hour with a small quantity of normal serum; and this extraction could be repeated as often as 6 times. Herbert and Todd (1944) showed that the properties of Weld's hæmotoxin were identical with those of streptolysin-S and that the hæmolysin was also extracted by lecitho-vitellin; hence they suggested that a lipo-protein fraction of serum was responsible for the extraction. In view of the results described above, the question arose whether peroxide influences the extraction of hæmolysin from streptococci. Five strains of hæmolytic streptococci of Lancefield's groups A, C, E, G and L were obtained from the National Collection of Type Cultures, and the point was tested by the following method. Twenty c.c. of meat-extract broth in a 4 × 1 inch container were inoculated with a hæmolytic streptococcus and incubated for 12-14 hours at 37° C. Longer incubation much diminished the yield of hæmolysin (*cf.* Weld, 1934). The culture was then centrifuged and the supernatant removed. Two c.c. of normal horse or sheep serum and several glass beads were added to the deposit and the mixture was shaken for one hour. The serum extract after separation by the centrifuge and the whole shaken mixture were equally hæmolytic, the M.H.D. being 0.01-0.005 c.c. If extraction was carried out with normal serum containing 1/10-1/20 volume hydrogen peroxide, hæmolysin was absent from the extract. The cultures were not sterilised during extraction, as plating showed a rich growth of hæmolytic colonies. In order to test the possibility that hæmolysin was extracted in an oxidised, inactivated form the peroxide-serum extracts were treated with a reducing agent—sodium sulphite or thioglycolic acid; no hæmolysin was found in the reduced extracts.

The following experiments demonstrated that peroxide-treated

hæmolytic organisms of Lancefield's groups A, C and E inhibited hæmolysin production by any of the strains of groups A, C, E, G and L. Naturally-occurring strains of *Strep. viridans* were also inhibitory, but generally to a less degree.

It is known that peroxide accumulates in cultures of streptococci grown in shallow layers of catalase-free media (McLeod and Gordon, 1923). Accordingly, a watery extract of catalase was prepared from pork (Euler, 1906). When 1 c.c. of this was added to 5 c.c. of serum broth and the mixture inoculated with a hæmolytic streptococcus along with an  $\alpha$  variant, the anti-hæmolytic action was abolished. This suggests that the anti-hæmolytic action of  $\alpha$  variants is due to their producing peroxide. Incidentally it was occasionally observed that a  $\beta$  strain when first tested in shallow layers of serum broth produced ample hæmolysin, but when a similar test was made a few days later the yield was greatly diminished. Such a result was always associated with the presence in the culture of an  $\alpha$  variant. When the strain was purified, a full yield of hæmolysin was again obtained.

*Effect of growth in filtrates of variant cultures on hæmolysin production by hæmolytic streptococci*

Cultures of  $\alpha$  variants grown for 12-14 hours at 37° C. in deep tubes containing horse-serum broth were Seitz-filtered. A mixture of filtrate with an equal volume of fresh horse-serum broth allowed, in general, good growth of  $\beta$  streptococci, but hæmolysin production was much reduced. When the variant culture was grown in a shallow layer of serum broth and incubated for 18 hours, the filtrate regularly inhibited hæmolysin production completely and in some cases inhibited growth of the organisms. Such filtrates when diluted with 4 parts of serum broth, inhibited hæmolysin production but did not inhibit growth of the organisms. All the above filtrates were catalase-free and contained peroxide approximately equivalent to 1/10-1/20 volume solution of hydrogen peroxide. It appeared that peroxide in the higher concentration inhibited growth of the hæmolytic organisms and consequently hæmolysin production. The addition of a preparation of catalase to the filtrate counteracted both inhibitory effects; on the other hand, boiled preparations in which the catalase had been destroyed showed neither action. In the lower concentration, peroxide inhibited hæmolysin production but did not affect growth of the organisms; in this case the addition of boiled, as well as fresh, catalase prevented the anti-hæmolytic action of the filtrate. This property of boiled catalase may possibly be due to its content of lipoids.

*Action of variant strains on filtered hæmolysin*

There is no evidence that variant strains destroy or inhibit the action of pre-formed hæmolysin. Seitz-filtered serum hæmolysin is

very labile at 37° C. When a mixture of equal parts of hæmolysin and serum broth was inoculated with a variant and tested after keeping for 3 and 6 hours at 37° C., it was not destroyed more rapidly than in a sterile control mixture of hæmolysin with fresh serum broth. Filtrates of variant cultures are actually less anti-hæmolytic than a control of uninoculated horse-serum broth, the slight inhibitory action of which is due to the serum. A mixture of streptolysin-S (Todd, 1938) with hydrogen peroxide to give a final concentration of 1/10 volume was allowed to stand for one hour at room temperature, after which it showed the same titre as a control with saline. Thus, in agreement with the results of Todd (1928) and Fuller and Maxted (1939), peroxide in the concentrations used does not inhibit the action of pre-formed hæmolysin.

*Effect of peroxide on the extraction by serum of hæmolysin from hæmolytic streptococci*

Weld (1934, 1935) extracted a powerful hæmotxin from the deposit of a centrifuged broth culture of a hæmolytic streptococcus by shaking for one hour with a small quantity of normal serum; and this extraction could be repeated as often as 6 times. Herbert and Todd (1944) showed that the properties of Weld's hæmotxin were identical with those of streptolysin-S and that the hæmolysin was also extracted by lecitho-vitellin; hence they suggested that a lipo-protein fraction of serum was responsible for the extraction. In view of the results described above, the question arose whether peroxide influences the extraction of hæmolysin from streptococci. Five strains of hæmolytic streptococci of Lancefield's groups A, C, E, G and L were obtained from the National Collection of Type Cultures, and the point was tested by the following method. Twenty c.c. of meat-extract broth in a 4 × 1 inch container were inoculated with a hæmolytic streptococcus and incubated for 12-14 hours at 37° C. Longer incubation much diminished the yield of hæmolysin (*cf.* Weld, 1934). The culture was then centrifuged and the supernatant removed. Two c.c. of normal horse or sheep serum and several glass beads were added to the deposit and the mixture was shaken for one hour. The serum extract after separation by the centrifuge and the whole shaken mixture were equally hæmolytic, the M.H.D. being 0.01-0.005 c.c. If extraction was carried out with normal serum containing 1/10-1/20 volume hydrogen peroxide, hæmolysin was absent from the extract. The cultures were not sterilised during extraction, as plating showed a rich growth of hæmolytic colonies. In order to test the possibility that hæmolysin was extracted in an oxidised, inactivated form the peroxide-serum extracts were treated with a reducing agent—sodium sulphite or thioglycolic acid; no hæmolysin was found in the reduced extracts.

The following experiments demonstrated that peroxide-treated

streptococci do not yield hæmolysin on subsequent extraction with serum. The sediment from a broth culture was shaken for one hour with serum containing 1/10 volume  $H_2O_2$ . This was centrifuged, the supernatant removed and tested for hæmolysin, and the deposited cells re-shaken with fresh serum. Neither extraction yielded any hæmolysin. Streptococci extracted twice with serum alone gave hæmolysins each with a M.H.D. of 0.005 c.c. Further, peroxide-treated bacteria when shaken a second time with serum *plus* isotonic catalase solution failed to yield hæmolysin. The accompanying table shows the results of such an experiment carried out with the sediment from a 15-hour culture of a group-G strain.

TABLE:

*Hæmolysin yielded by two successive extractions of hæmolytic streptococci*

Culture: portion no.	First extraction		Second extraction	
	Extractor	Hæmolysin (M.H.D. in c.c.)	Extractor	Hæmolysin (M.H.D. in c.c.)
1	2 c.c. serum	0.005	2 c.c. serum + 1 c.c. saline	0.01
2	2 c.c. serum + 1/10 vol. $H_2O_2$	None *	2 c.c. serum + 1 c.c. saline	None
3	2 c.c. serum + 1/10 vol. $H_2O_2$	None	2 c.c. serum + 1 c.c. catalase	None

\* None = hæmolysin absent from 0.5 c.c.

Similar experiments in which catalase was replaced by the reducing agents sodium sulphite or thioglycolic acid yielded the same result. Further, it was found that streptococci shaken with serum containing 1/10 volume hydrogen peroxide, allowed to stand dry at 5° C. for 48 hours and finally extracted with serum did not yield hæmolysin, although this was found in control cells shaken with serum alone and then treated similarly.

Thus organisms treated with concentrations of peroxide which did not exert a marked killing action nevertheless failed to yield hæmolysin on subsequent extraction with serum. But treated organisms returned to the hæmolytic state when plated on blood agar.

## DISCUSSION

Todd (1928) first observed that disappearance of hæmolysin from cultures of hæmolytic streptococci coincided with the appearance of peroxide, but he concluded that the phenomena were unrelated, since peroxide had no action on hæmolysin. Fuller and Maxted, who

confirmed these findings, noted in addition that cultures of  $\alpha$  variants produced peroxide earlier than the hæmolytic organisms. They concluded that peroxide acted as an antiseptic and by preventing multiplication of the organisms inhibited hæmolysin production. A difficulty arose, however, in reconciling the amount of peroxide required to inhibit hæmolysin production with the much greater amount required to prevent multiplication of the organisms.

Since the work of Weld on serum extraction of streptococcal hæmolysin, a new conception of its nature has arisen. Hæmolysin is looked upon as a normal component of the cellular complex of the hæmolytic streptococcus, which can be extracted from the organisms by serum. The formation of serum hæmolysin in serum-broth cultures of streptococci thus involves two stages—the production of hæmolysin within the cell membrane of proliferating streptococci and the extraction of the hæmolysin by a lipo-protein fraction of the serum. The process is presumably similar on blood agar.

The results described above accord with this hypothesis. The anti-hæmolytic action of  $\alpha$  variants is due to their production of peroxide. Further, the addition of peroxide to a culture of a hæmolytic streptococcus in concentrations not exceeding that produced during the growth of an  $\alpha$  variant prevents subsequent extraction of hæmolysin from the hæmolytic organisms. The variant strains of Todd (1928) and Fry (1933), which were hæmolytic anaerobically and non-hæmolytic aerobically, may be regarded as intermediate in character between hæmolytic streptococci and *Strep. viridans*, the peroxide produced in aerobic growth being sufficient to render the cells, as it were, impermeable to hæmolysin; thus they would appear "non-hæmolytic". The  $\alpha$  variants here described, like the naturally-occurring *Strep. viridans*, appear to represent a further stage in this process. Such an explanation implies that the capacity for hæmolysin production has not been lost by the variants; hence from the genetic point of view there would be less difficulty in accounting for cases in which the hæmolytic form reappears. But the question remains unanswered whether *viridans* organisms have ceased to produce hæmolysin or whether it is produced in a form so far unextractable.

An almost constant feature of the  $\alpha$  variants was that the change to the non-hæmolytic character was accompanied by a change in the culture from rough to smooth. In addition, unlike the "intermediate" strains of Todd and of Fry, the variants had lost the carbohydrate group-specific antigen, or it had become modified. It remains to be determined whether these simultaneous changes have the same mechanism.

### Summary

1. The anti-hæmolytic action of  $\alpha$  variants of hæmolytic streptococci is due to their producing peroxide and can be abolished by adding catalase to the medium.

2. Hæmolytic streptococci treated with non-lethal concentrations of hydrogen peroxide similar to those produced in cultures of  $\alpha$  variants no longer yield hæmolysin on extraction with serum, but they can give rise to hæmolytic descendants.

Thanks are due to the Rankin Medical Research Fund for a grant towards the expenses of this work.

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# BULLET EMBOLISM: A CASE OF PULMONARY EMBOLISM FOLLOWING THE ENTRY OF A BULLET INTO THE RIGHT VENTRICLE OF THE HEART

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(PLATE XXXII)

EMBOLISM resulting from the movement of a bullet or other projectile within the blood vessels is a rare but not unrecorded event. Only eight cases of pulmonary embolism of this nature have been found in the literature. Impaction of foreign bodies in systemic arteries or veins has been commoner. The behaviour of solid metallic bodies lying free in the heart or blood channels is a matter of interest to the morbid anatomist and yet only one paper on the subject (Straus, 1942) has appeared in a pathological journal within recent years, although there have been a number of records of the surgical removal or radiological observation of such bodies. The object of this paper, therefore, is to describe a new case of pulmonary embolism by a bullet and to present a short review of the literature relating to foreign body embolism.

## CASE REPORT

### *Clinical history*

Male, aged 38, an aircraftsman employed as a fitter at a R.A.F. station in England. Family history of mental instability. Depression and anxiety for about a year, culminating in a carefully prepared attempt on his own life on 21st November 1944. The weapon was of his own make and consisted of a solid block of aluminium about the size and shape of a matchbox through which he had drilled a hole of the right calibre to receive a Sten gun cartridge. A metal clip attached the contraption to his braces, holding the nose of the bullet directed towards the heart. He fired the shot by striking the breech of the cartridge forcibly with a heavy spanner wielded by his right hand supporting his own body against the trunk of a tree. The bullet entered his chest. There was very little bleeding or shock and he drank some Lysol but promptly spat it out. He then walked away to seek help.

### *Clinical examination and course*

On admission to Wharnccliffe Emergency Hospital, Sheffield, some four hours later, he was vomiting but was able to walk into the ward and undress. There was a small wound of the chest over the heart but no exit wound, and signs



of a small left pleural effusion were present. X-ray showed a bullet within the thorax, localised as being intra-cardiac by screening and cross-direction plates. It moved with the heart beat but did not whirl about as though it were free in the blood currents. High swinging fever commenced on the next day and persisted till death. On 29th November pericardium and heart wall were explored at operation but the bullet was not detected. The left hæmothorax, which was infected, was drained. Thereafter he was very ill. Fever was uncontrolled by chemotherapy. It was regrettable that no further X-ray examination could be made. He died on 10th December, having survived his injury for 19 days.

### *Post-mortem examination*

*External.* Wasted adult male. Small healed puncture wound on front of left chest, level with and  $1\frac{1}{2}$  in. medial to the nipple and overlying the 4th intercostal space. No burning or powdering of skin. Healed surgical incision on precordium. Sores on lips consistent with attempt to drink corrosive liquid.

*Internal.* A segment of the 4th and 5th ribs had been removed at operation. Infection beneath skin at operation site. *Pericardium.* Recent fibrinous pericarditis with about 2 oz. of turbid exudate. *Heart.* The heart was first opened *in situ* and was further dissected after removal with the rest of the thoracic viscera. On the anterior surface a small tear in the muscle corresponded in position to the wound of entry in the overlying skin. This point of entry into the heart was situated on the anterior wall of the right ventricle just to the right of the anterior interventricular sulcus and at the base of the conus arteriosus. On opening the heart, a deep ragged ulcer,  $16 \times 6$  mm. in diameter, was exposed on the right ventricular aspect of the interventricular septum. It lay just below the left anterior cusp of the pulmonary valve (fig. 1) and was lined by blood clot and torn muscle fibres. No bullet was found in the heart, and indeed the rest of the organ appeared normal. *Lungs.* Septic left hæmothorax. Blood-stained effusion in right pleural sac. The middle and lower lobes of the right lung felt solid and on section gave the appearance of a hæmorrhagic infarct, being drier and firmer than the rest of the lungs, which were œdematous. There were slight pigmentary changes and septic softening in portions of the infarct, which was clearly not agonal. The bullet lay in the lower primary branch of the right pulmonary artery, pointing down the lower lobe branch and occluding the middle lobe branch by lying across its orifice. It filled the artery where it lay and firm thrombus extended from its apex into the distal parts of the vessels. A loose propagated clot lay just behind the bullet (fig. 2). The bullet weighed 7.45 g. and measured 9.0 mm. in calibre and about 14.5 mm. in length. It was found to fit snugly, when placed nose downwards, into the ulcer crater on the interventricular septum of the heart. *Other organs* showed no evidence of disease other than passive venous congestion of the abdominal viscera.

## BULLET EMBOLISM



FIG. 1.—Heart. Right ventricle opened to show the inter-ventricular septum, with a deep ulcer where the bullet had lodged just below the left anterior cusp of the pulmonary valve.



FIG. 2.—Right lung lower lobe and portion of middle lobe. Hæmorrhagic infarction with pigmentary changes and septic softening. The bullet is impacted in a branch of the pulmonary artery and is surrounded by blood clot.



*Comment*

It was evident that the bullet had been discharged at very low velocity since the improvised weapon possessed no breech block to absorb the counter-thrust of the cartridge explosion. In its short course it had traversed the man's shirt and vest, skin, chest wall, intercostal tissues, left pleura, anterior border of left lung, pericardium and anterior heart wall, crossed the conus arteriosus of the right ventricle and become embedded in the interventricular septum. Hæmorrhage from the lung resulted in a left hæmothorax which quickly became infected. The bullet narrowly missed the anterior descending branch of the left coronary artery and there was no bleeding into the pericardial sac. It is not known when the bullet escaped its lodgement in the septum and became free in the right ventricle, nor when it passed into the pulmonary artery to cause infarction of the right lower and middle lobes. The patient had been very ill, with delirium and high fever, for many days before dying from the combined effects of sepsis in the left pleura and pericardium and of the extensive pulmonary infarction.

## DISCUSSION

Wounds of the heart are not uncommon. The three important causes of early death in these cases are hæmorrhage, cardiac tamponade and disturbance of heart function (King, 1941). Where there is neither an open wound nor progressive intrapericardial bleeding the patient will usually make a good immediate recovery, even although a foreign body is lodged within the heart (Turner, 1941). The arrest of a missile in the heart results from its low velocity, most often in the case of a spent bullet but sometimes as a result of the bullet striking a hard object on its passage, and occasionally, as here, when an imperfect weapon is used. There have been many instances of missiles lodging in the pericardium. These need not be further discussed. There are also a few records of projectiles remaining embedded in cysts or scar tissue within the heart wall (Gilchrist, 1929). Turner (1940) observed a man with a bullet in the wall of the left ventricle for 23 years. Foreign bodies have been located on various occasions in each of the four chambers of the heart, but they have been found far more frequently on the right side than on the left. There are two reasons for this. First, the heart is usually wounded from the front and the anterior aspect of the heart exposes about twice as much of the right as of the left ventricle, in addition to the right auricle and root of the pulmonary artery. Elkin (1941) found in a series of 38 cases, mainly stab-wounds, that the right ventricle was more often wounded (17 instances) than any other chamber or intrapericardial vessel. Second, foreign bodies may be carried as emboli in the systemic veins to reach the right side of the heart (*e.g.* Lyle, 1917; Bland-Sutton, 1919).

In these circumstances it is remarkable that pulmonary embolism from the ejection of an intra-cardiac missile into the pulmonary artery has been so rare. Decker (1939-40), reviewing 109 cases of foreign body in the heart and pericardium reported in the literature since 1900, states that only 2 out of 27 deaths were attributed to pulmonary embolism, although 39 foreign bodies reached the right ventricle and 8 the right auricle. It is also apparent that the embolic movement of missiles is much less common in the pulmonary artery than in other blood vessels. Paltauf (1933), surveying 31 records of projectile embolism, found that only 3 affected the pulmonary artery, and Straus (1942) found only 4 of this kind in his 32 collected cases. So far as can be determined, having regard to the imperfect bibliography of some of the publications, only eight instances of pulmonary embolism by bullet or shell fragment have hitherto been recorded. Decker (1939-40) quotes two cases, one described by Maffi in 1917 and one by Moore in 1932. Straus refers to two earlier cases reported by Morestin in 1903 and by Hoffmann prior to 1912. Four other cases (Fullerton, 1919; Fry, 1920; Paltauf, 1933; Straus, 1942) may be given fuller mention as they show some of the different ways in which pulmonary embolism can occur.

Fullerton observed immediate signs of pulmonary infarction in a soldier wounded in the left loin. At autopsy he was able to see that the bullet, taking an upward and forward course, had passed through the posterior wall of the right ventricle and been swept into the upper main branch of the right pulmonary artery. Fry records the migration of a rifle bullet from the left internal iliac vein through the right auricle and ventricle to the lower lobe branch of the left pulmonary artery. This bullet on entry had produced a communication between the vein and the external iliac artery and the consequent rise in venous pressure propelled the bullet towards the heart. It is well known that a metallic embolus may fall by gravity against the normal venous current. This has been observed radiologically (Grandgérard, 1917), when a shrapnel ball, whirling about in the right auricle, suddenly dropped down the inferior vena cava and came to rest in the right hypogastric vein. Davey and Parker (1946-47), operating to remove a shell fragment from the left innominate vein, observed it to slip first into the right auricle and thence down the inferior vena cava into the right iliac vein. The influence of gravity on the movement of metallic bodies in the vessels is also seen in Straus's case of fatal pulmonary embolism. This patient, a negro, was wounded in the abdomen, the bullet entering the right common iliac vein. He was brought to hospital sitting up in a taxi and Straus thinks that the bullet moved to the heart and thence to the left pulmonary artery when the patient was put to bed in a head-down posture to combat shock. Harken and Williams (1946) write of two successful operations for the removal of foreign bodies from the pulmonary arteries. In one of these a bullet which was in the left artery slipped into the main

vessel and then into the right artery while the patient was lying on his right side. Paltauf's case of pulmonary embolism differs from the others in that the bullet entered directly into the intrapericardial portion of the pulmonary artery and moved on to cause infarction of the lower lobe of the right lung.

As in the present case death was not always due simply and directly to pulmonary infarction. In Straus's case there was also general peritonitis, in Paltauf's pericardial tamponade and in Fry's toxæmia from gangrene of the leg.

A word may be said about systemic arterial embolism by projectiles. Occasionally these enter by way of a wound of the left ventricle (O'Neill, 1917), sometimes by a wound of a large artery such as the iliac or femoral but more commonly by penetration of the thoracic or abdominal aorta as in 10 of the 32 cases reviewed by Straus. Penetrating bullet wounds of the aorta are not always fatal (Bland-Sutton). Sometimes the bullet, having entered the ascending arch of the aorta, falls back into the left ventricle: more usually it is swept towards the periphery to become impacted in a vessel of appropriately diminished calibre.

The general conclusions to be drawn from the foregoing may be stated as follows. Solid missiles which enter the vascular system may migrate therein in a direction which is determined by the balance between the effects of gravity and the propelling pressure of the blood stream. Projectiles are more likely to enter the right-sided than the left-sided chambers of the heart, and may arrive there by direct wound of the heart by a low velocity missile or by carriage through the veins. Pulmonary embolism by the projectile is a somewhat rare sequel and is not by itself necessarily a direct cause of death, even though the embolus and resulting area of pulmonary infarction are large.

### SUMMARY

A case is recorded of pulmonary embolism by a bullet which became free within the right ventricle after having been temporarily embedded in the interventricular septum.

The subject of embolism by intravascular missiles is briefly reviewed: only eight previous cases of pulmonary embolism have been found in the literature.

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# THE PATHOGENESIS OF GLOMERULO-NEPHRITIS: A RE-INVESTIGATION OF THE AUTO-IMMUNISATION HYPOTHESIS

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THE resemblance of the lesions observed in human glomerulo-nephritis to those produced experimentally by injection into animals of antisera against renal tissue prepared in animals of another species (Smadel, 1936, *lit.*) has stimulated numerous attempts to obtain antibodies against renal tissue in animals of the same species.

The first successful steps in this direction were reported by Schwentker and Comploier (1939), who obtained in-vitro evidence of complement-fixing antibodies after the repeated injection into rabbits of rabbit kidney emulsion plus staphylococcus toxin. In 1945 Cavelti and Cavelti (*a, b* and *c*) reported the successful production of auto-antibodies to kidney tissue in rabbits and rats, and described clinical and pathological changes in the rats closely resembling those found in human acute and chronic glomerulo-nephritis. Their best results were obtained by the repeated intraperitoneal injection of group-A streptococci (grown on synthetic medium and killed by ether, alcohol, chloroform or heat) mixed with finely ground fresh rat kidney tissue which had been freed from blood by perfusion with saline *in vivo*. Dosage of the mixture of 10 per cent. kidney tissue suspension plus 1 per cent. streptococcus suspension varied from a total of 0.5 c.c. to 20 c.c., and courses varied from a single injection to injections repeated on ten consecutive days. Antibodies were detected by the collodion particle agglutination technique, using fresh kidney extract to sensitise the particles. In their experiments, urinary changes (increased proteinuria, casts and red cells) usually appeared 7-14 days after the end of the course of immunisation—a period of time corresponding roughly to that which elapsed before antibodies were detected. The incidence of urinary changes following single injections of relatively large doses was low, but with repeated injections totalling 20 c.c. the incidence was 100 per cent. Animals which showed no effects after a first course of injections often responded when the course was repeated one month or more later. The antibody titre was not closely correlated with the intensity of the urinary changes, and maximum antibody production tended to appear after longer courses of immunisation than were necessary for the production of urinary changes.

In control experiments in which kidney tissue alone was injected neither antibody formation nor nephritis resulted; when streptococci alone were used only occasional positive results were obtained, and these only with the largest doses.



Cavelti and Cavelti (1945a) concluded that their evidence supported the suggestion previously advanced by Schwentker and Comptoir that human glomerulo-nephritis may be due to auto-antibodies to kidney cells. They postulated that during the acute streptococcal infection which commonly precedes acute nephritis a complex kidney-streptococcus antigen is formed which initiates the formation of auto-antibodies to kidney tissue. Acute glomerulo-nephritis is precipitated by the subsequent reaction of the antibodies with the kidney cells.

This hypothesis, should it prove correct, has important implications for the study of diseases other than glomerulo-nephritis, for example rheumatic fever. Although the ætiology of rheumatic fever is unknown, it is a disease which is closely associated with streptococcal infection and its histopathology resembles that found in experimental allergic conditions. Furthermore it has recently been observed by Hawn and Janeway (1947) that when single injections of purified bovine plasma-protein fractions are given intravenously to rabbits, the lesions in animals which show a hypersensitive response may predominantly involve either the large arteries and heart valves or the glomeruli, depending upon which fraction is injected.

In order to investigate the part played by streptococci in the causation of rheumatic fever it was desirable to have a reliable biological indication of their power to evoke auto-antibodies, and it was decided to attempt to repeat the Caveltis' work. No evidence in support of these claims could be obtained. This discrepancy is so puzzling and calls so urgently for elucidation that the following investigation is reported in some detail.

#### MATERIALS AND METHODS

*Rats.* The Medical Research Council inbred strain of black and white hooded rats was used. The animals were housed in cages arranged so that every cage contained one member of each experimental group. Some cages contained females but the majority were males. The experiment was conducted during winter and spring, but the animal room was maintained at a temperature of 60°-70° F. Diet consisted of compressed rat cubes made to the formula of Parkes (1945-46), and water was given *ad lib.* On this diet the rats grew steadily during the experiment from an initial weight of 120-150 g. to 200-250 g. They were able to bear and to rear full litters.

All the rats were infested to a slight degree with *Capillaria*, the ova of which were found in the urine. *Acarus* infestation was also present, but was controlled by the occasional application of benzyl benzoate emulsion. Neither of these parasites appeared to have any adverse effect on the health of the rats.

*Urine.* This was collected over 24-hr. periods during which the rats received no food or water. During the 24 hrs. preceding each period of collection they were given a solution of 0.3 per cent.  $\text{NH}_4\text{Cl}$  + 0.1 per cent.  $\text{NaCl}$  in water to drink, in order to acidify the urine, as recommended by the Caveltis (1945c). The urine was preserved from bacterial growth during collection by the addition of a few drops of 6 per cent. formaldehyde. About half of the centrifuged deposit was examined for cellular constituents. Protein was estimated in the supernatant with Tsuchiya's reagent as described by the Caveltis and the results expressed in mg. per 24 hrs.

**Kidneys.** These were obtained from normal rats after perfusion with sterile 0.9 per cent. NaCl solution via the left auricle under ether anaesthesia. Perfusion was continued until the kidneys were blanched and macroscopically free from blood: microscopically a few red cells could still be seen in fixed sections. The kidneys were removed with aseptic precautions, blotted and weighed. They were then ground very finely with sand and saline in a chilled mortar and, after brief centrifugation to remove large particles, the suspension was stored at  $-76^{\circ}\text{C}$ . It was diluted with physiological saline before use to give a 10 per cent. suspension calculated from the wet weight of the kidney material. Such a suspension contained 2.0-2.2 per cent. kidney solids. All batches of kidney were tested for sterility before being stored at  $-76^{\circ}\text{C}$ .

**Autolysed kidney.** Fifty per cent. suspensions of fresh kidney in saline were gently shaken with toluene and incubated under toluene at  $37^{\circ}\text{C}$ . for 24 hrs. The toluene was removed *in vacuo* and the autolysate stored at  $+2^{\circ}\text{C}$ . Such autolysates were sterile and showed no whole cells in stained films.

**Streptococci.** Two strains were used, both belonging to Lancefield group A but differing widely in virulence.

**Strain "Richards" (N.C.T.C. 5631).** This strain had been passaged through mice and was highly virulent (M.L.D. for mice by intraperitoneal inoculation 5-25 organisms). It produced powerful streptolysin "O" and "S". It was grown on the semi-synthetic medium of Bernheimer *et al.* (1942) supplemented with 0.2 per cent. marmite, since growth was slow in the original medium. Rather large inocula had to be used and there was some reduction in virulence (M.L.D. for mice approximately 1000 organisms). Growth was allowed to proceed at  $37^{\circ}\text{C}$ . for 48-60 hrs. and the acid produced was neutralised by the addition of NaOH after 24 and 48 hrs.

The organisms were centrifuged out and, after removal of the supernatant, re-suspended in saline as a 2 per cent. (v/v) suspension. Ether\* was added to 10 per cent. (v/v) and after violent shaking the mixture was allowed to stand for 24 hrs. at  $2^{\circ}\text{C}$ . The ether was then removed by the pump and the suspension of organisms stored at  $-76^{\circ}\text{C}$ . Tests of sterility were made by inoculation of 1 c.c. or more of a 2 per cent. suspension into 100 c.c. of nutrient broth and by spreading a loopful of bacterial deposit over a blood-agar plate. It was found that, unless the streptococcal suspension had been shaken violently with ether, some organisms survived.

**Strain N.Y. 5.** This strain was obtained from Dr P. A. Cavelti. It was relatively avirulent to mice (M.L.D. approximately  $10^4$  organisms), although it produced a powerful haemolysin. It gave a similar growth to strain "Richards" on synthetic medium, but was mostly grown on a semi-synthetic medium consisting of an acid hydrolysate of casein with yeast extract + marmite and glutamine, and 1 per cent. glucose. Growth on this medium at  $37^{\circ}\text{C}$ . was excellent. Acid produced was neutralised at 24, 36 and 48 hours and the organisms were then harvested. Subsequent treatment was the same as that of the "Richards" strain.

**Streptococcus-kidney mixtures.** One volume of a 2 per cent. saline suspension of killed organisms was mixed with 1 volume of 20 per cent. kidney suspension. The mixture was allowed to stand at room temperature (approximately  $20^{\circ}\text{C}$ .) for at least half-an-hour before being injected, but the maximum period of exposure to room temperature of any mixture was 4 hours. The Caveltis (1945b) tried precipitation of the streptococcus-kidney mixture with acetone at  $70^{\circ}\text{C}$ . in order to obtain a dry powder, but since they found that a simple mixture was equally effective only this method was used.

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\* The ether used was later tested and found to contain peroxides. The total quantity of peroxides in 10 c.c. of ether was equivalent to 0.015 mg. of  $\text{H}_2\text{O}_2$ —a quantity so small as to be unlikely to alter the streptococci significantly.

*Schedule of immunisation*

Two schedules were used, in one of which the rats received single large injections of streptococci, of kidney suspension or of both, and in the other repeated smaller doses. Schedule 1 consisted of a single intraperitoneal injection of 10 c.c. repeated after 2 months; schedule 2a of 5 intraperitoneal injections of 2.5 c.c. at 2-day intervals, repeated after 2 months; schedule 2b of 9 intraperitoneal injections of 2.5 c.c. at daily intervals.

*General plan of experiment*

Groups of rats were injected with different materials as follows:—

(A) 8 rats received streptococcus "Richards" kidney mixtures according to schedule 1 and 8 according to schedule 2a.

(B) 10 rats received streptococcus N.Y. 5 kidney mixtures according to schedule 2b.

(C) 6 rats received streptococcus "Richards", 1 per cent. in saline, according to schedule 1 and 6 according to schedule 2a.

(D) 6 rats received kidney suspension, 10 per cent. in saline, according to schedule 1 and 6 according to schedule 2a.

(E) 8 rats were used as cage controls and received no inoculations.

*Collodion particle agglutination*

Collodion particles were prepared by the method of Cannon and Marshall (1910) and were separated by differential centrifugation as described by Cavelti (1914). The technique of sensitisation of particles with antigen used by Cavelti was simple admixture of diluted particles with a suitable dilution of antigen immediately before use. This method proved satisfactory, but it was found that more dependable results were obtained when the particles were coated by allowing a thick suspension of particles to stand in contact with a strong solution of antigen at 2° C. overnight. The sensitised particles were then diluted 100-fold with 0.45 per cent. NaCl before use, giving final dilutions corresponding to those used by Cavelti. All tests were controlled with normal serum and with unsensitised particles. The method was tested with three known systems:

(a) Particles sensitised with a 1:500,000 solution of purified type I pneumococcus capsular polysaccharide were agglutinated by a 1:42,000 dilution of commercial anti-pneumococcus type I rabbit serum.

(b) Two rabbits were injected intraperitoneally with 5 × 1 g. of frog muscle suspension. Sera from these rabbits caused specific agglutination of particles coated with muscle extract to a titre of 1:1024. The optimal dilution of frog muscle extract for sensitising the particles was a 1:40 to 1:60 dilution of a 20 per cent. extract.

(c) A rabbit was injected intraperitoneally with 4 × 1 g. rat-kidney suspension and test-bled at intervals. Its serum agglutinated particles coated with rat-kidney extract to a maximum titre of 1:1980 and with autolysed rat-kidney extract to a maximum titre of 1:360. The optimal dilution of rat-kidney extract for sensitising the particles was 1:80 of a 10 per cent. extract.

The technique of collodion particle agglutination performed with tissue extracts appeared to be 5-10 times more sensitive than the complement fixation technique, using 2 M.H.D. of complement.

## RESULTS

*Urinary examinations*

The urine of all the rats was examined on one or more occasions before injections were commenced and every four days during a period of three weeks after a course of injections. During the remainder of the experiment, which lasted four months, they were examined weekly. The results are summarised in the table. They show no significant

TABLE

*Summary of urine examinations on immunised and control rats*

Group	Injected with	No. of rats		No. of urinary examinations	Mean protein (mg. per 24 hrs.)	Percentage of urines examined containing		
		♂	♀			casts	red cells	leuco-cytes
A	Streptococci and kidney .	10	6	240	4.3	5	10	5
B	" " " " .	0	10	165	2.3	1	11	3
C	Streptococci " " .	8	4	161	5.9	4	11	4
D	Kidney " " " " .	12	0	154	6.8	4	5	2
E	Normal " " " " .	3	5	113	4.0	4	5	6
F	Group E plus pre-injection urines from rats of groups A-D	33	25	161	3.5	3	5	6

differences between any of the groups and the normal rats. Even when casts were present they were usually very scanty (2-10 per 24 hrs.) and never exceeded 40 per 24 hrs.

It was observed incidentally that protein excretion tended to increase with age, and that male rats excreted more protein (average 6.5 mg./24 hrs. per rat; range 4.2-9.9 mg.) than females (average 2.5 mg./24 hrs. per rat; range 1.6-5.5 mg.).

*Serological examinations*

The rats were bled from the heart at the beginning of the experiment, at 10-15 days and 24 days after the end of the first course of injections, and at 9-11 days and 30 days after the end of the second course of injections. Sera were stored at +2° C. without preservative and were mostly tested within a fortnight of bleeding.

Tests were made on 22 sera taken from rats of groups A and B (kidney and streptococci), after the first course of injections and on 24 sera taken after the second course. Thirteen sera from rats of group C (streptococci only) and 12 from group D (kidney only) were tested only after the second course of injections. Neither by the collodion particle agglutination technique, using fresh and autolysed kidney extracts at the concentrations found to be optimal in the model experiments referred to above, nor by the complement fixation technique, were any unequivocal anti-kidney antibodies detected.

*Autopsy studies*

The rats were killed 6 weeks after the end of the second course of injections. Macroscopically all organs other than the kidneys were normal, except for enlarged mesenteric lymph glands in 60 per cent. of those which received injections containing kidney tissue. The kidneys also were normal except in one rat of group C, in which they were enlarged and pale. With this exception the weight of kidney per 100 g. carcass of the experimental groups lay within the same limits as those of 15 control rats.

*Histology*

Sections of kidney stained with hæmatoxylin and eosin were examined from all the rats. None showed changes suggestive of glomerulo-nephritis, although sections from the rat of group C mentioned above showed the picture of pyelonephritis. Patchy lymphocytic infiltration around interlobular vessels and in the adipose tissue of the calyces was found in 18 animals, but this finding was not confined to any particular group and appeared equally in the controls. It is a condition found not uncommonly in laboratory rats and reference is made to it by Jaffé (1931).

*Additional experiments with autolysed kidney*

It was shown by Lewis (1933, 1941) and by Schwentker and Rivers (1934) that alcoholic extracts of rabbit brain or autolysed rabbit brain emulsion were effective as antigens in rabbits, whereas fresh brain emulsion appeared not to be so. Hecht, Sulzberger and Weil (1943), who claim to have produced antibodies to skin in rabbits by injection of homologous skin suspension and staphylococcal toxin, found that their sera fixed complement with autolysed rather than with fresh rabbit skin. It seemed likely that, even if small amounts of antibody were formed against surface components of homologous tissue cells, they would be fixed by these cells so rapidly as to escape detection in the plasma, whereas antibodies against internal constituents might remain in the circulation for longer periods. In view, therefore, of the negative findings obtained in the previous experiments it was decided to attempt to obtain antibodies against renal tissue by the use of autolysed kidney suspensions.

Seven rats which had previously received 20-25 c.c. each of fresh kidney-streptococcus mixture intraperitoneally were therefore given two courses of 10 per cent. autolysed kidney+1 per cent. streptococcus "Richards" suspension, each course consisting of 5 intraperitoneal injections of 2 c.c. at 2-day intervals, with an interval of 3 weeks between the courses. The urine was examined weekly for a period of 9 weeks, and sera were taken 14 days after the first course and

6 and 21 days after the second. Urine and sera were tested as before, but neither urinary changes nor antibodies to fresh or autolysed kidney extract were detected.

### DISCUSSION

In view of the unequivocal nature of Cavelti and Cavelti's claims the results obtained were surprising. So far as possible their technique, as described, was rigidly adhered to, and although the number of rats used was much smaller than in their experiments, the doses of kidney-streptococcus mixture were chosen to be at least as large as those which produced 100 per cent. incidence of intense urinary changes in their animals. In these experiments, therefore, some rats at least should have shown corresponding changes. Some also should have yielded demonstrable anti-kidney antibodies, even if the technique of testing for them were less sensitive than in the Caveltis' hands.

In seeking for an explanation of the discrepancy various possibilities have been considered. It is well known (*e.g.* Kenton, 1941) that rats are in general poor producers of detectable antibodies and that the Arthus phenomenon can rarely be elicited in these animals except when passive immunisation is performed. The Evans strain of rats, largely used by the American workers, may possess unusual ability to produce antibodies. The development of nephritis might be dependent upon the activation of some latent renal infection by trauma to the kidney consequent upon the immunising injections, but in that case it would be difficult to account for the Caveltis' negative results in control groups of rats which received either streptococci or kidney suspensions alone or streptococcus-rat heart mixtures. Again, the ability to become sensitised to auto-antigens may be associated with dietary factors in a similar way to the variations described by Sulzberger and Mayer (1931) in the readiness with which guinea-pigs can be sensitised to intracutaneous injections of neo-arsphenamine. It was clearly not possible to supply diets identical with those on which the Caveltis' rats were fed, but perusal of the main constituents (private communication from Dr P. A. Cavelti) does not show any obvious differences in type of diet.

The general principles underlying the production of auto-antibodies by means of injections of bacteria plus homologous tissues have such far-reaching implications that it seemed important to record failure to repeat clear-cut results under apparently well defined conditions. If the Caveltis' work can be confirmed elsewhere an analysis of unnoticed differences in technique may give an answer to why some animals react while others do not—a question which is itself of considerable significance. For this reason the description of the methods used in these experiments is given more fully than might otherwise have seemed necessary.

## SUMMARY

An attempt has been made to repeat the observation of Cavelti and Cavelti that intraperitoneal injection into rats of streptococci plus rat kidney suspensions is followed by the development of antibodies to rat kidney and of glomerulo-nephritis in the rats. Using black and white hooded rats these results have not been confirmed.

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## IMMUNOLOGICAL STUDIES WITH GROUP-B STREPTOCOCCI

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THE low virulence of many strains of *Streptococcus agalactiae* for laboratory animals appears to have proved an obstacle to immunological studies with this organism, and attempts to protect cows against *Str. agalactiae* infection of the udder by means of vaccines have so far been unsuccessful.

Griffith (1935), however, in his study of the human Aronson streptococcus demonstrated that mice could be immunised successfully against the Aronson N strain by gradually increasing intraperitoneal doses of a young, heat-killed, glucose-broth culture of the homologous strain, and that immunity was still demonstrable 15 days after the last immunising dose. He was unable to produce similar immunity by subcutaneous vaccination and failed to demonstrate immunity to the human Aronson S strain in mice immunised against the Aronson N strain. He mentioned that his demonstration of the possibility of actively immunising mice against the Aronson N strain confirmed earlier work by Yoshioka (1922-23) and Killian (1924*a* and *b*). In addition to demonstrating active immunity in mice to the Aronson N strain, Griffith reported that an efficient protective serum against this strain could be prepared in the rabbit; an intraperitoneal dose of 0.14 ml. of this serum protected mice against as much as 0.05 ml. of virulent culture with an M.L.D. of  $10^{-9}$  ml.

In her work on the serological classification of hæmolytic streptococci, Lancefield (1933) examined both the Aronson S strain (O 89 in her series) and the Aronson N strain (O 90 in her series) and found that O 89 belonged to her group A and O 90 to her group B. In a later publication (Lancefield, 1934) she reported that two of her group-B strains, O 90 of human origin and K 158 A from a laboratory rabbit, were sufficiently virulent for use in mouse-protection tests. With antiserum prepared in rabbits, she demonstrated that mice could be protected against 10-100 M.L.D. of cultures of the homologous type, but not against a lethal dose of cultures of heterologous types.

On the other hand, Francis (1947) failed to immunise mice against a group-B streptococcus of bovine origin. The mice were inoculated subcutaneously with living avirulent organisms or with heat-killed cultures, and immunity was tested by intraperitoneal inoculation with a mouse-passaged homologous strain. Because this procedure showed no evidence that the mice had been protected Francis concluded that *Str. agalactiae* was incapable of stimulating immunity.

Stableforth (1946) reviewed the literature on the serological classification of streptococci within Lancefield's group B, and pointed out that serological differences may be associated with differences in the capacity to confer cross protection after immunisation.



The present paper presents evidence that mice and guinea-pigs can be actively immunised with living or formolised cultures against group-B streptococci of human and bovine origin, and records the results of experiments designed to examine the immunological relationships between serologically different group-B streptococci. The object of the work is to elucidate some of the problems involved in field vaccination of cattle against mastitis due to *Str. agalactiae*.

#### MATERIALS AND METHODS

**Strains.** Nine strains of serologically identified group-B streptococci were used, 7 bovine and 2 human. All showed  $\beta$ -haemolysis on blood agar, and were sodium hippurate positive and aesculin negative; all fermented glucose, lactose, sucrose and salicin, but not mannitol, inulin, raffinose or sorbitol; all except bovine strain S 90 fermented trehalose; all produced acid and clot in litmus milk, and their final pH in glucose broth ranged between 4.0 and 4.5. The close biochemical similarity between strains of bovine and human origin has been previously noted by several workers, e.g. Pomaies-Lebrón, *et al.* (1947). The origin, year of isolation, serological type and pathogenicity for mice of the various strains are given in table I. None of the strains inoculated as described killed all the mice in every experiment.

TABLE I

*Origin, year of isolation, serological type, and pathogenicity for mice of seven bovine and two human strains of group-B streptococci*

Strain	Origin	Year of isolation	Source	Serological type	Pathogenicity for mice: 0.5 ml 1p of broth culture containing not less than 100 million viable organisms per ml.			
					No. of expts.	Total mice inoculated	Survivors	
							No.	Per cent.
S 1	Bovine	1946	Teat sore	1 ? subtype	4	39	13	33
S 2	"	1913	Mastitis	3a	9	52	51	98
S 3	"	1946	Teat sore	1 ? subtype	7	74	9	12
S 4	"	1946	"	"	3	34	5	15
S 13	"	1943	Mastitis	"	59	747	172	23
S 15	Human	1946	Puerperal fever	?	9	161	19	12
S 21	"	1946	"	?	6	91	11	12
S 90	Bovine	1945	Mastitis	1 ? subtype	3	34	2	6
S 117	"	1945	"	"	3	34	4	12

**Media.** Six media were used:—(i) Hartley's broth prepared as described by Mackie and McCartney (1942, p. 101), except that the final pH was adjusted to 7.4 instead of 7.6. (ii) Walbum's broth prepared as described by Mackie and McCartney (1942, p. 330). (iii) Todd and Hewitt's broth, batch 1; the medium described by Todd and Hewitt (1932), but sterilised by steaming at 100° C. on three successive days instead of by filtration. (iv) Todd and Hewitt's broth, batch 3; the medium described by these authors. (v) Todd and Hewitt's broth, batch 1, plus 20 per cent. sheep serum. This is referred to as serum broth. (vi) Glucose broth prepared as for Hartley's broth plus 0.5 per cent. glucose and the final pH adjusted to 7.2.

White Swiss mice from a closed population, each weighing 13.22 g., and guinea-pigs from a closed population, each weighing 200-500 g., were used;

in any one experiment test and control animals were of the same sex and of comparable weight. A standard inoculum of 0.5 ml., measured by a 1.0 or 2.0 ml. syringe, was used in all experiments with living cultures.

The number of viable organisms in a culture was determined by a surface plate count on blood agar. Dilutions in normal saline were prepared at  $10^{-2}$ ,  $10^{-4}$ , and  $10^{-6}$ , with dropping pipettes standardised to deliver 0.02 ml. drops. Three separate series of dilutions were always made, so that the resulting count was the mean of three counts, each made from a dilution independent of the other two. All cultures were examined microscopically for purity before inoculation.

All mice that died were examined bacteriologically, or, if the number was so great as to make this impracticable, a random sample of the dead mice was examined.

In most of the tables the time is not given at which animals were recorded as surviving the test dose. This time was 5 days, since experience showed that deaths at a later date were exceptional among mice that were healthy 5 days after intraperitoneal inoculation. The great majority died within 36 hours of inoculation.

To simplify the tables and for the sake of brevity statistical analysis of the results of all experiments has been omitted. It should be understood, however, that any inference drawn from the figures given is based on statistical tests.

### EXPERIMENTAL OBSERVATIONS

#### *Increase of survival time to bovine strain S 13*

In an experiment to determine the virulence of bovine strain S 13 grown overnight in Hartley's broth, it was noted that all of 29 mice inoculated intravenously with the standard dose of 0.5 ml. died within ten days, but that when the same dose was given intraperitoneally to 69 mice there were 18 survivors.

It was decided, therefore, to test survivors from the intraperitoneal inoculation for resistance to the lethal intravenous dose. In the first test, 10 mice which had survived 58-62 days after intraperitoneal

TABLE II

*Results of intravenous inoculation with bovine strain S 13 of : (A) mice previously inoculated intraperitoneally with living cultures of the same strain ; (B) untreated mice*

Expt. no.	Group. (No. of days between 1st and 2nd inoculation)	No. of mice	No. of deaths at days nos.				Survivors	
			1-5	6-10	11-15	Over 15 (day of death)	No.	Well at day no.
1	A. Previously inoculated (58-62 days)	10	1	5	3	1 (48)	0	...
	B. Untreated . . . . .	10	10	0	0	0	0	...
2	A. Previously inoculated (32 days)	7	0	4	0	1 (27)	2	77
	B. Untreated . . . . .	9	8	1	0	0	0	...

inoculation were used and 10 mice not previously inoculated served as controls. The result (table II) suggested a longer survival time in mice that had received the intraperitoneal inoculation. To confirm

this a further seven mice which were healthy 32 days after intraperitoneal inoculation were tested with the lethal intravenous dose. The result of this test (table II) again showed that previously inoculated mice survived longer than control mice not previously inoculated.

### *Acquired immunity to human strain S 15*

These findings showed that intraperitoneal inoculation of bovine strain S 13 produced some degree of resistance to the same strain inoculated intravenously. It was decided, therefore, to test whether resistance could be so stimulated by another strain, and whether it could be built up still further by gradually increasing intraperitoneal doses.

For this purpose human strain S 15 grown overnight in serum broth was used, four intraperitoneal inoculations at 4-day intervals being given as the immunising course to a group of 20 mice. The four inoculations consisted of 0.01, 0.05, 0.1, and 0.2 parts of the standard inoculum of 0.5 ml. made up to 0.5 ml. with normal saline. The use of serum broth produced anaphylaxis in some of the mice, and of the 20 inoculated 8 died of shock during the course of injections.

Human strain S 15 grown overnight in serum broth was used as the test dose, and 0.5 ml. was inoculated intraperitoneally four days after the last immunising inoculation. The result is given in table III.

TABLE III

*Results of intraperitoneal inoculation with human strain S 15 of: (A) mice previously inoculated with living culture of the same strain; (B) untreated mice*

Expt. no.	Group. (No. of days between last immunising inoculation and test dose)	No. of mice	Death in		Survivors
			less than 4 hours (anaphylactic shock)	1-5 days	
1	A. Previously inoculated (4 days) .	12	0	2	10
	B. Untreated . . . . .	20	0	16	4
2	A. Previously inoculated (4 days) .	20	6	4	10
	B. Untreated . . . . .	20	0	20	0

When the experiment was repeated, only one of the 30 immunised mice died of anaphylactic shock before the test dose was given. The test dose was again 0.5 ml. of human strain S 15 in serum broth, inoculated intraperitoneally. In this experiment (table III) 6 of the previously inoculated mice died of anaphylactic shock within four hours of the test inoculation.

These experiments showed that immunised mice acquired considerable resistance to the test dose.

*Duration of immunity to bovine strain S 13 and persistence of living organisms in immunised mice*

An experiment was next arranged to determine the duration of immunity after a series of inoculations of living bovine strain S 13 and how long living streptococci persisted in immunised mice. A group of 172 mice was inoculated intraperitoneally at 4-day intervals, as before, with bovine strain S 13 grown overnight in Todd and Hewitt's broth, batch 1. Groups of 12 of these mice were then tested for immunity on days 2, 6, 10, 14, 18, 24, 30, 36, 42, 48 and 54 after the last immunising inoculation. The remaining 40 of the 172 immunised mice were not given the test dose but were killed for bacteriological examination of the heart blood, liver, spleen and kidney on days 1, 2, 3, 4, 5, 6, 10, 14, 18, 24, 30, 36, 42, 48 and 54 after the last immunising inoculation. Cultures on blood agar were examined after 24 and 48 hours' aerobic incubation at 37° C.

The results of this experiment (tables IV and V) showed that in every test there were deaths among the immunised mice and survivors

TABLE IV

*Duration of immunity in mice inoculated intraperitoneally at 4-day intervals with living cultures of bovine strain S 13. (Test dose 0.5 ml. intraperitoneally of homologous strain)*

Expt. no.	Test no.	Days after last immunising inoculation	No. of mice in each group		No. of survivors in each group	
			Immunised	Control	Immunised	Control
1	1	2	12	12	11	5
	2	6	12	12	10	2
	3	10	12	12	7	4
	4	14	12	12	4	1
	5	18	12	12	10	1
	6	24	12	12	9	4
	7	30	12	12	9	2
	8	36	12	12	9	4
	9	42	12	12	10	2
	10	48	12	12	5	3
	11	54	12	12	10	5
2	1	4	12	12	9	3
	2	29	12	12	7	3
	3	59	12	12	3	0
	4	64	56	56	16	0

among the controls, but the number of survivors in the immunised group was invariably higher than in the corresponding untreated group. Taking the groups as a whole, immunity was as good at the end of the 54-day period as at the beginning. The low survival rates in the immunised mice of tests 4 and 10 could arise from chance alone. This part of the experiment was repeated, the groups being tested at wider intervals (table IV). This second experiment had been planned to last some 6 months, and enough mice had been inoculated to allow

groups of 12 to be tested at intervals of about a month. After the third test, however, an outbreak of ectromelia among mice in the same room as the immunised animals made it imperative that they should be tested and discarded as soon as possible. For this reason the fourth test followed only 5 days after the third, and 56 mice were tested. The low survival rate (16 of 56) confirmed an impression formed after the third test of the second experiment, in which only 3 of 12 immunised mice survived, that immunity was being lost in the group as a whole. However, even this figure was in line with that of the other tests in that survival among the immunised mice was significantly higher than among the non-immunised controls. Table V shows that streptococci were isolated sporadically up to but not beyond the tenth day after the final immunising inoculation.

TABLE V

*Results of bacteriological examination of heart blood, liver, spleen, and kidney of 40 mice inoculated intraperitoneally at 4-day intervals with immunising doses of living cultures of bovine strain S 13*

Days after last immunising inoculation	No. of mice examined	Streptococci isolated from
1	2	Kidney of one mouse
2	3	No organ
3	2	Heart and spleen of one mouse
4	2	No organ
5	2	"
6	3	"
10	3	Heart of one mouse (only heart and kidney cultured)
14	3	No organ
18	3	"
24	3	"
30	3	"
36	3	"
42	3	"
48	3	"
54	2	"

During this experiment, post-mortem examination of immunised mice killed for bacteriological examination without being given the test dose, showed in many of them an apparent enlargement of the spleen. To estimate the extent of this enlargement, the spleen weight as a percentage of body weight was determined for 12 immunised and 12 untreated mice. The results (table VI) show that the groups overlapped slightly, but on the average the index in immunised mice was about  $2\frac{1}{2}$  times that in untreated mice.

*Immunisation with bovine strain S 13 grown in Walbum's medium*

For this experiment bovine strain S 13 was grown overnight in Walbum's broth. After this period, growth in this medium was dense, but from previous experience it was known that 0.5 ml. of

this culture given intraperitoneally would be sub-lethal to the majority of the mice. Twenty-two mice were therefore inoculated intraperi-

TABLE VI

*Spleen weight as percentage of body weight of: (A) mice previously inoculated at 4-day intervals with immunising doses of living cultures of bovine strain S 13; (B) untreated mice*

A. Previously inoculated mice (12)			B. Untreated mice (12)		
Spleen weight (g.)	Body weight (g.)	Spleen weight as percentage of body weight	Spleen weight (g.)	Body weight (g.)	Spleen weight as percentage of body weight
0.20	19.3	1.00	0.04	17.5	0.23
0.18	18.0	1.00	0.03	19.0	0.16
1.10	12.9	0.77	0.08	18.0	0.44
0.19	16.8	1.10	0.03	17.0	0.17
0.12	15.9	0.75	0.10	17.0	0.59
0.08	13.8	0.58	0.16	21.0	0.76
0.24	18.5	1.30	0.08	18.5	0.43
0.17	17.8	1.00	0.05	16.0	0.31
0.35	16.5	2.10	0.08	17.5	0.46
0.17	20.5	0.85	0.13	17.5	0.74
0.27	23.5	1.10	0.05	16.0	0.31
0.14	16.0	0.88	0.02	15.0	0.13
Range 0.58-2.10 per cent. Average 1.03 per cent.			Range 0.13-0.76 per cent. Average 0.39 per cent.		

toneally four times at 4-day intervals with this dose, and were tested for evidence of immunity 5 days after the last immunising inoculation with a test dose of 0.5 ml. intraperitoneally of the same strain grown overnight in Todd and Hewitt's broth, batch 1. All the 22 immunised mice (table VII) survived the test dose, but 17 of 22 untreated mice

TABLE VII

*Results of intraperitoneal inoculation with 0.5 ml. of bovine strain S 13 grown overnight in Todd and Hewitt's broth, batch 1, of: (A) mice previously inoculated 4 times at 4-day intervals with a sub-lethal dose of the same strain (0.5 ml. of overnight culture in Walbum's broth); (B) untreated mice*

Group. (No. of days after last immunising inoculation)	No. of mice	Survivors
A. Previously inoculated (5 days)	22	22
B. Untreated	22	5
A. Re-test of previously inoculated survivors (75 days)	22	9
B. Untreated	22	0

succumbed. Seventy-five days after this test, the 22 immunised survivors were again exposed to a test dose of bovine strain S 13. As shown in table VII, some of the mice had lost their immunity but others retained it.

*Immunisation with mixed cultures of bovine strain S 13,  
human strain S 15 and human strain S 21*

It is generally agreed that immunity to *Str. agalactiae* infection in cattle has not so far been produced by vaccination, but the possibilities of achieving successful vaccination have not yet been exhausted. Further, if immunity is related to serological type, a vaccine against bovine mastitis should contain several different strains of streptococci. To determine if mice could be immunised at the same time against more than one strain of group-B streptococci, 3 strains were used: bovine strain S 13, human strain S 15 and human strain S 21.

For each immunising inoculation, the three strains were grown separately and the pooled inoculum was prepared immediately before inoculation. A group of 75 mice was immunised, the animals being inoculated intraperitoneally at 4-day intervals as follows. Day no. 1, 0.5 ml. of pooled inoculum prepared from an overnight growth in glucose broth of each strain diluted 1:5 with normal saline before pooling; day no. 5, the same diluted 1:2; day no. 9, the same undiluted; day no. 13, 1.0 ml. of pooled inoculum prepared as for day no. 9; day no. 17, 0.5 ml. of pooled inoculum prepared from an overnight undiluted growth of each strain in Todd and Hewitt's broth, batch 3.

Six days after the final immunising inoculation the mice were divided into three groups and inoculated intraperitoneally with a test dose of 0.5 ml. of a 6-hour glucose broth culture of either bovine strain S 13, human strain S 15, or human strain S 21. The results (table VIII) showed that a considerable degree of immunity had been developed against all three strains.

TABLE VIII

*Results of inoculation of: (A) mice immunised with mixed inocula of bovine strain S 13, human strain S 15, and human strain S 21; (B) untreated mice, with a test dose (0.5 ml. of a 6-hour glucose-broth culture) of one or other of the 3 homologous strains*

Group. (No. of days after last immunising inoculation)	Test strain	No. of mice	No. of survivors
A. Previously inoculated (6 days) . . . . .	Bovine S 13	25	25
B. Untreated . . . . .	"	25	11
A. Previously inoculated (6 days) . . . . .	Human S 15	25	23
B. Untreated . . . . .	"	25	3
A. Previously inoculated (6 days) . . . . .	Human S 21	25	24
B. Untreated . . . . .	"	25	5
A. Re-test of previously inoculated human S 15 survivors (41 days)	Human S 15	20	1
B. Untreated . . . . .	"	20	1

Forty-one days after this test, 20 of the 23 survivors from the group tested against human strain S 15 were still healthy, 3 having died of intercurrent disease. These 20 were again tested against human strain S 15 but immunity could no longer be demonstrated (table VIII).

*Immunisation with formolised cultures of bovine strain S 13*

So far immunisation had been accomplished by intraperitoneal inoculation of gradually increasing doses of living organisms. In view of the inconvenience of handling living cultures and the difficulty of standardising inocula, I decided to try killed cultures.

After 6 hours in glucose broth, bovine strain S 13 showed dense growth with 300 million viable organisms per ml. Formalin was then added to give a final concentration of 0.3 per cent. The flask of culture was incubated for one hour more and then placed in the cold store; examination next day showed that the organisms had been killed.

In the first experiment 12 mice were inoculated intraperitoneally with 0.5 ml. of this formolised whole culture on three consecutive days, and after an interval of four days, on two more consecutive days. Eight days after the last immunising dose they were inoculated intraperitoneally with a test dose of 0.5 ml. of the same strain grown overnight in Todd and Hewitt's broth, batch 3.

In the second experiment the same formolised culture of bovine strain S 13 was used as vaccine after cold storage for 17 days. Fifteen mice were immunised by intraperitoneal inoculation with 0.5 ml. of the vaccine, and thereafter on four more occasions at 2-day intervals with 1.0 ml. Five days after the last immunising dose they were exposed to a test dose of 0.5 ml. of bovine strain S 13 grown overnight in Todd and Hewitt's broth, batch 3.

Results of these two experiments (table IX) indicate that immunity against the homologous strain was as good after immunisation with

TABLE IX

*Results of intraperitoneal inoculation of 0.5 ml. of bovine strain S 13 grown overnight in Todd and Hewitt's broth, batch 3, of: (A) mice previously immunised with formolised whole culture of the same strain; (B) untreated mice*

Expt. no.	Group. (No. of days after last immunising inoculation)	No. of mice	Survivors
1	A. Previously inoculated (8 days) . . . . .	12	11
	B. Untreated . . . . .	12	3
2	A. Previously inoculated (5 days) . . . . .	15	14
	B. Untreated . . . . .	15	0

formolised whole culture as with gradually increasing doses of living organisms, and that the formolised whole culture retained its antigenic properties for at least 17 days in cold storage.



To determine how few immunising inoculations would confer some protection, a fresh formolised vaccine was prepared from an overnight culture in Todd and Hewitt's broth, batch 3, with 250 million viable organisms per ml. Groups of mice were inoculated intraperitoneally with this vaccine as follows: (i) 1 inoculation of 1.0 ml., (ii) 2 inoculations of 1.0 ml. with a 2-day interval between inoculations, (iii) 3 inoculations of 1.0 ml. at 2-day intervals, (iv) 4 inoculations of 1.0 ml. at 2-day intervals, (v) 5 inoculations at 2-day intervals.

Eight days after the last inoculation the mice were exposed to a test dose of 0.5 ml. intraperitoneally of bovine strain S 13 grown overnight in Todd and Hewitt's broth, batch 3. None of the mice that had received only one immunising inoculation survived the test dose, but there was evidence of protection in all other groups (table X).

TABLE X

*Results of intraperitoneal inoculation with 0.5 ml. of bovine strain S 13 in Todd and Hewitt's broth, batch 3, of: (A) mice previously inoculated intraperitoneally with one or more doses of formolised whole culture of the same strain; (B) untreated mice*

Group. (No. of days after last preparatory inoculation)	No. of immunising inoculations	No. of mice	No. of survivors
A. Previously inoculated (8 days) . . . . .	1	7	0
B. Untreated . . . . .		11	0
A. Previously inoculated (8 days) . . . . .	2	11	7
B. Untreated . . . . .		11	0
A. Previously inoculated (8 days) . . . . .	3	15	11
B. Untreated . . . . .		15	1
A. Previously inoculated (8 days) . . . . .	4	14	9
B. Untreated . . . . .		14	2
A. Previously inoculated (8 days) . . . . .	5	14	12
B. Untreated . . . . .		14	2

Statistical examination showed that 2 immunising inoculations gave as good protection as 5. None of the immunisation regimes protected all mice.

The number of mice in the various groups of this experiment ranged from 7 to 15, because an outbreak of ectromelia killed mice in all groups except no. iii. The untreated mice, however, had also been exposed to ectromelia infection and were accordingly regarded as adequate controls.

#### *Immunisation of guinea-pigs against bovine strain S 13*

To determine if guinea-pigs could also be immunised four 200-g. guinea-pigs were given 4 intraperitoneal inoculations at 2-day intervals of living cultures of bovine strain S 13 grown overnight in Todd and

Hewitt's broth, batch 3, in doses of 0.5, 0.5, 1.0, and 1.5 ml. After an interval of 5 days 3 further intraperitoneal inoculations of the same strain grown in the same medium were given at 2-day intervals in doses of 3.0, 4.0, and 5.0 ml. Two days after the last immunising inoculation, these four animals and two untreated animals were exposed to an intraperitoneal test dose of 6.0 ml. of the homologous strain grown overnight in Todd and Hewitt's broth, batch 3. One of the 4 treated guinea-pigs and both the untreated died within 24 hours, and streptococci were isolated from the heart, liver and spleen of all 3 animals. The three remaining immunised guinea-pigs showed no evidence of disease and were discarded some 2 months later.

This indicated that immunisation of guinea-pigs by methods similar to those used with mice might be possible, and the following experiment was carried out. Bovine strain S 13 grown for 6 hours in glucose broth provided 350 million viable organisms per ml. and the culture was formolised (0.3 per cent.) as before.

Fourteen 500-g. guinea-pigs were then inoculated intraperitoneally with formolised culture at 4-day intervals with 2.0, 3.0 and 5.0 ml. Four days after the last inoculation with formolised culture they were inoculated intraperitoneally with 2.0 ml. of living culture of bovine strain S 13 grown overnight in Todd and Hewitt's broth, batch 3. Four days after the last immunising dose they were inoculated, together with 15 untreated guinea-pigs of comparable weight, with a test dose of 3.0 ml. intraperitoneally of the homologous strain grown overnight in Todd and Hewitt's broth, batch 3. Ten of the 15 untreated animals died, but all the immunised animals survived (table XI).

TABLE XI

*Results of intraperitoneal inoculation with 3.0 ml. of bovine strain S 13 grown overnight in Todd and Hewitt's broth, batch 3, of : (A) guinea-pigs inoculated intraperitoneally with a series of doses of formolised and living cultures of bovine strain S 13 ; (B) untreated guinea-pigs*

Group. (No. of days after last immunising inoculation)	No. of guinea-pigs	No. of survivors
A. Previously inoculated (4 days)	14	14
B. Untreated	15	5

Post-mortem examination of the dead guinea-pigs showed dark, gelatinous subcutaneous tissue, intense congestion of the mesenteric vessels and vessels of the abdominal wall, hæmorrhages over the colon, excess of dirty peritoneal fluid, and excess of pleural and pericardial fluid. Stained smears showed that the peritoneal and pleural fluids were swarming with streptococci, and streptococci were isolated from heart-blood, liver, spleen and kidney. These post-mortem findings were similar to those noted in deaths from the

peritoneal form of *Str. pneumoniae* type XIX infection. Since the guinea-pig colony from which these animals came was known to be carrying this infection, the streptococci isolated from four of the animals killed experimentally were examined and appeared to be biochemically identical with the streptococci inoculated—bovine strain S 13.

*Passive immunity in mice, using serum from immunised guinea-pigs*

It seemed probable that antibodies against bovine strain S 13 would be present in the serum of the immunised guinea-pigs used in the preceding experiment and might be detectable by mouse-protection tests. Four days after exposure to the test dose, the guinea-pigs were bled by cardiac puncture and their sera pooled. Normal guinea-pig serum was obtained from two healthy, untreated adults. All sera were kept in the cold store without preservative.

Lancefield (1934) stated that type-specific protection was effective with group-B streptococci when infecting organism and specific immune serum were injected simultaneously, whereas with group-A organisms it was necessary to inject the antiserum at least 8 hours before the infecting organism in order to demonstrate protection. My first attempt to demonstrate protective antibodies against bovine strain S 13 in the pooled sera of immunised guinea-pigs was therefore made by mixing an overnight growth of this strain in Todd and Hewitt's broth, batch 3, with an equal quantity of serum, incubating for 20 minutes at 37° C. and inoculating mice intraperitoneally with 1.0 ml. of the mixture (*i.e.* 0.5 ml. of culture and 0.5 ml. of serum). Mice inoculated with 1.0 ml. of a mixture of equal parts of culture and of serum from untreated guinea-pigs served as controls.

All mice in both groups died (table XII). Sufficient serum from both immunised and untreated guinea-pigs remained after this

TABLE XII

*Results of intraperitoneal inoculation of mice with 1.0 ml. of bovine strain S 13 grown overnight in Todd and Hewitt's broth, batch 3, mixed in equal parts with pooled sera from (A) guinea-pigs immunised against the homologous organism; (B) untreated guinea-pigs*

Group	No. of mice	No. of survivors
A. Test organism + serum from immunised guinea-pigs	10	0
B. Test organism + serum from untreated guinea-pigs	10	0

experiment to attempt passive protection of a small number of mice by intraperitoneal inoculation of serum first and of the test dose 24 hours later. In this trial 1.0 ml. of serum was used—twice the

amount used in the first attempt. The results (table XIII) suggested some degree of protection.

TABLE XIII

*Results of intraperitoneal inoculation with 0.5 ml. of bovine strain S 13 grown for 6 hours in glucose broth of mice inoculated intraperitoneally 24 hours before with 1.0 ml. of pooled sera from (A) guinea-pigs first immunised against and later exposed to a test dose of the homologous organism; (B) untreated guinea-pigs*

Expt. no.	Group	No. of days after exposure of guinea-pigs to test dose	No. of mice	No. of survivors
1	A. Mice previously inoculated with serum from immunised guinea-pigs	4	6	4
	B. Mice previously inoculated with serum from untreated guinea-pigs	...	4	1
2	A. Mice previously inoculated with serum from immunised guinea-pigs	13	15	8
	B. Mice previously inoculated with serum from untreated guinea-pigs	...	10	0

This result was confirmed by bleeding the same guinea-pigs a second time, 13 days after exposure to the test dose of bovine strain S 13. Their sera were again pooled. Serum for control mice was obtained by pooling sera from four untreated guinea-pigs. Mice were inoculated intraperitoneally with 1.0 ml. of pooled serum from the immunised guinea-pigs, and control mice with 1.0 ml. of pooled serum from untreated guinea-pigs. Twenty-four hours later both groups were exposed to an intraperitoneal test dose of 0.5 ml. of bovine strain S 13 grown for six hours in glucose broth (table XIII). Eight of 15 mice previously inoculated with serum from the immunised guinea-pigs survived the test dose; all of 10 control mice died.

*Examination of nine strains of group-B streptococci for evidence of cross immunity*

In all the experiments so far described, immunised animals were tested for evidence of immunity by exposure to a test dose of the homologous strain or strains, and three strains only were used—bovine S 13, human S 15 and human S 21. Cross-immunity tests were therefore carried out with the same three strains and six additional bovine strains.

Mr P. Stuart of the Ministry of Agriculture and Fisheries' Veterinary Laboratory, Weybridge, kindly examined the strains serologically (table I) and classified bovine strain S 2 as serological type 3a, and all the other bovine strains as serological type 1 ? subtype. He reported further that the six bovine strains designated type 1 ? subtype fell into the main type 1 described by Stableforth (1937), but that

none was identical with any of Stableforth's British subtypes, although all were closely related to subtype 1b, while the human strains S 15 and S 21 were serologically distinct from all the bovine strains and from each other.

The first cross-immunity experiment was carried out with bovine strain S 13 and the two human strains S 15 and S 21. Forty-five mice were inoculated intraperitoneally at 4-day intervals with 0.01, 0.05, 0.1, and 0.2 parts of the standard inoculum (0.5 ml.) of human strain S 21 grown overnight in serum broth and made up to 0.5 ml. with normal saline. Four days after the last immunising inoculation they were exposed in batches of 15, together with an equal number of untreated mice, to test doses of 0.5 ml. of bovine strain S 13, human strain S 15 and human strain S 21, each grown overnight in Todd and Hewitt's broth, batch 1. The results (table XIV) showed significant

TABLE XIV

*Results of intraperitoneal inoculation with bovine strain S 13, human strain S 15 and human strain S 21 of: (A) mice previously immunised against human strain S 21; (B) untreated mice*

Expt. no.	Group. (No. of days between last immunising inoculation and test dose)	Test strain	No. of mice	No. of survivors
1	A. Previously inoculated (4 days)	Bovine S 13	15	12
	B. Untreated . . . . .	"	15	6
2	A. Previously inoculated (4 days)	Human S 15	15	5
	B. Untreated . . . . .	"	15	5
3	A. Previously inoculated (4 days)	Human S 21	15	11
	B. Untreated . . . . .	"	15	2

protection against the homologous strain ( $p = \text{less than } 0.001$ ), but not against human strain S 15. Protection against bovine strain S 13 was only of low significance ( $p = 0.03$ ) and further experiments failed to show any relationship between it and human S 21.

In the second cross-immunity experiment 72 mice were immunised against bovine strain S 13 (type 1 ? subtype), and were then exposed in groups of 12 to test doses of bovine strains S 4, S 13, S 90, S 1, S 3 and S 117 (all type 1 ? subtype). Each test dose was 0.5 ml. of an overnight growth in Todd and Hewitt's broth, batch 3, given four days after the last immunising inoculation, which was accomplished by intraperitoneal inoculation at 4-day intervals of: (i) 1.0 ml. of formalised whole culture, prepared by adding 0.3 per cent. formalin to a 6-hour glucose-broth culture; (ii) 1.0 ml. of the same formalised whole culture; (iii) 0.5 ml. of an overnight growth in Todd and Hewitt's broth, batch 3, diluted 1 : 2 with normal saline; (iv) 0.5 ml. of undiluted overnight growth in Todd and Hewitt's broth, batch 3. The results of this experiment (table XV) showed significant protection of immunised mice against all strains.

Six days after this experiment, immunised mice which had survived the various test doses were again tested for evidence of immunity, this time against bovine strains S 3 and S 13 (both type 1 ? subtype), and against human strain S 15 (of different serological type). Test

TABLE XV

*Results of intraperitoneal inoculation of: (A) mice immunised against bovine strain S 13 (type 1 ? subtype); (B) untreated mice, with 0.5 ml. of an overnight growth in Todd and Hewitt's broth, batch 3, of six bovine strains, all of the same serological type as the immunising strain*

Group	No. of mice	Test strain	No. of survivors
A. Immunised . . . .	12	S 4	11
B. Untreated . . . .	12	"	3
A. Immunised . . . .	12	S 13	12
B. Untreated . . . .	12	"	0
A. Immunised . . . .	12	S 80	12
B. Untreated . . . .	12	"	0
A. Immunised . . . .	12	S 1	12
B. Untreated . . . .	12	"	0
A. Immunised . . . .	12	S 3	12
B. Untreated . . . .	12	"	1
A. Immunised . . . .	12	S 117	12
B. Untreated . . . .	12	"	1

doses were in each case 0.5 ml. intraperitoneally of an overnight growth in Todd and Hewitt's broth, batch 3. There was still strong immunity to bovine strains of the same type, but no significant immunity to the human strain (table XVI).

TABLE XVI

*Results of intraperitoneal inoculation of: (A) mice immunised against bovine strain S 13 (type 1 ? subtype) that survived a test dose of the homologous organism; (B) untreated mice, with 0.5 ml. of an overnight growth in Todd and Hewitt's broth, batch 3, of bovine strain S 3, bovine strain S 13, or human strain S 15*

Group	Immunised against	Previously survived test dose of	Now exposed to	No. of mice	No. of survivors
A. Immunised . . . .	Type 1 ? subtype	Type 1 ? subtype	Type 1 ? subtype	18	17
B. Untreated . . . .	...	...	"	18	3
A. Immunised . . . .	Type 1 ? subtype	Type 1 ? subtype	Human S 15	17	4
B. Untreated . . . .	...	...	"	17	0
A. Immunised . . . .	Type 1 ? subtype	Type 1 ? subtype	Type 1 ? subtype	18	17
B. Untreated . . . .	...	...	"	18	1
A. Immunised . . . .	Type 1 ? subtype	Type 1 ? subtype	Human S 15	18	3
B. Untreated . . . .	...	...	"	18	2

These cross-protection tests indicated that: (i) human strain S 21 had no demonstrable immunological relationship with human strain S 15; (ii) six bovine strains of the same serological type were able to confer protection against each other; (iii) there was no demonstrable cross protection between human strain S 15 and the six bovine strains of serological type 1 ? subtype.

To verify that the six bovine strains of the same serological type conferred protection against each other, mice were immunised against bovine strain S 3 with two 1.0 ml. intraperitoneal inoculations of formalised whole culture and two inoculations of living culture at 4-day intervals, and were then exposed five days after the last immunising inoculation to 0.5 ml. intraperitoneal test doses of the same six bovine strains. The results (table XVII) confirmed the previous finding.

TABLE XVII

*Results of intraperitoneal inoculation of: (A) mice immunised against bovine strain S 3 (type 1 ? subtype); (B) untreated mice, with 0.5 ml. of an overnight growth in Todd and Hewitt's broth, batch 3, of six bovine strains of the same serological type*

Group	No. of mice	Test strain	Serological type of test strain	No. of survivors
A. Immunised	12	S 4	Type 1 ? subtype	12
B. Untreated	12	"	"	1
A. Immunised	12	S 13	"	9
B. Untreated	12	"	"	1
A. Immunised	12	S 90	"	10
B. Untreated	12	"	"	0
A. Immunised	12	S 1	"	12
B. Untreated	12	"	"	0
A. Immunised	12	S 3	"	10
B. Untreated	12	"	"	1
A. Immunised	12	S 117	"	11
B. Untreated	12	"	"	2

Six days after exposure to the test cultures in this experiment, the surviving mice were exposed to test doses of 0.5 ml. of overnight cultures in Todd and Hewitt's broth, batch 3, of human strains S 15 and S 21. The results (table XVIII) confirmed the previous observation that there was no cross protection between human strain S 15 and bovine strains of type 1 ? subtype, and showed a lack of cross protection between human strain S 21 and bovine strains of type 1 ? subtype.

Compared with the other strains, bovine strain S 2, serological type 3a, was of low virulence for mice when inoculated in the manner and dose described (table I). For this reason it could not be used as

a test strain. However, a group of mice was immunised against this strain by four intraperitoneal inoculations at 4-day intervals of (i)

TABLE XVIII

*Results of intraperitoneal inoculation of: (A) mice immunised against bovine strain S 3 (type 1 ? subtype) which had survived a test dose 6 days previously of a bovine strain of the same serological type; (B) untreated mice, with 0.5 ml. of human strain S 15 or human strain S 21, grown overnight in Todd and Hewitt's broth, batch 3*

Group	Test strain	No. of mice	No. of survivors
A. Immunised . . . .	Human S 15	15	1
B. Untreated . . . .	"	15	0
A. Immunised . . . .	Human S 21	16	1
B. Untreated . . . .	"	16	0
A. Immunised . . . .	Human S 15	13	1
B. Untreated . . . .	"	13	0
A. Immunised . . . .	Human S 21	17	3
B. Untreated . . . .	"	17	0

1.0 ml. of formolised 6-hour glucose broth culture; (ii) 0.5 ml. of an overnight glucose-broth culture; (iii) 0.5 ml. of a 6-hour glucose broth culture; and (iv) 0.5 ml. of an overnight growth in Todd and Hewitt's broth, batch 3. Four days after the last immunising inoculation the mice were exposed to test doses of bovine strains S 13 and S 3, both type 1 ? subtype, and to human strains S 15 and S 21.

The results (table XIX) showed that immunisation had conferred protection against all four test strains. This observation was confirmed by a second experiment (table XIX), so that it may be concluded that the bovine type 3a strain, S 2, was antigenic in spite of its low pathogenicity and immunologically distinct from the other six bovine strains, since mice were protected by it against test doses of both human strains. Further, this bovine type-3a strain was immunologically distinct from both human strains, since mice were protected by it against the other bovine strains.

#### DISCUSSION AND SUMMARY

Throughout these experiments the number and volume of intraperitoneal inoculations and the intervals between them were intentionally varied in order to examine the range in size of inoculum and frequency of inoculation required to produce detectable immunity in mice against group-B streptococci. The results indicated that detectable immunity was easily produced and that the volume of inoculum, number of inoculations and interval between inoculations might be varied within wide limits. Consistent results followed the



use of 4 intraperitoneal inoculations at 4-day intervals as follows :  
(i) and (ii) 1.0 ml. of formolised whole culture ; (iii) 0.5 ml. of over-

TABLE XIX

*Results of intraperitoneal inoculation of : (A) mice immunised against bovine strain S 2 (type 3a) ; (B) untreated mice, with 0.5 ml. of an overnight growth in Todd and Hewitt's broth, batch 3, of bovine strain S 3 (type 1 ? subtype) ; bovine strain S 13 (type 1 ? subtype) ; human strain S 15 ; or human strain S 21*

Expt. no.	Group. (Days after last immunising inoculation)	No. of mice	Tested against	No. of survivors
1	A. Immunised (4 days) .	11	Bovine S 3 (type 1 ? subtype)	9
	B. Untreated . . .	11	" " "	1
	A. Immunised (4 days) .	11	Bovine S 13 (type 1 ? subtype)	9
	B. Untreated . . .	11	" " "	2
	A. Immunised (4 days) .	11	Human S 15	8
	B. Untreated . . .	11	"	1
	A. Immunised (4 days) .	11	Human S 21	9
	B. Untreated . . .	11	"	0
2	A. Immunised (4 days) .	12	Bovine S 3 (type 1 ? subtype)	12
	B. Untreated . . .	12	" " "	0
	A. Immunised (4 days) .	12	Bovine S 13 (type 1 ? subtype)	9
	B. Untreated . . .	12	" " "	0
	A. Immunised (4 days) .	12	Human S 15	9
	B. Untreated . . .	12	"	0
	A. Immunised (4 days) .	12	Human S 21	12
	B. Untreated . . .	12	"	0

night glucose-broth culture ; (iv) 0.5 ml. of overnight culture in Todd and Hewitt's broth. The intraperitoneal test dose was given 4-6 days after the last immunising inoculation. The experimental results may be summarised thus :—

1. Mice previously inoculated intraperitoneally with a single dose of bovine strain S 13 showed a longer survival time than untreated mice when exposed to a lethal intravenous dose of the homologous organism.

2. Significant immunity to intraperitoneal test doses of the homologous strains of group-B streptococci of bovine and human origin was produced by intraperitoneal inoculation of a series of gradually increasing doses of living cultures.

3. Mice previously inoculated with a series of intraperitoneal doses of living cultures of bovine strain S 13, human strain S 15 and human strain S 21, given simultaneously, showed significant resistance to intraperitoneal test doses of all three strains.

4. Significant immunity to an intraperitoneal test dose of bovine strain S 13 was produced by intraperitoneal inoculation of a series of doses of formolised whole culture of the same strain.

5. Guinea-pigs previously inoculated intraperitoneally with a series of living cultures, or a series of formolised and living cultures, of bovine strain S 13 showed significant resistance to an intraperitoneal test dose of the same strain, and protective antibodies to bovine strain S 13 were detectable by mouse-protection tests in the pooled sera of immunised guinea-pigs up to at least 13 days after the last immunising inoculation with the same strain.

6. Cross protection could not be demonstrated between two human strains of different serological type, or between these two human strains and any of six bovine strains of serological type 1 ? subtype.

7. Cross protection was demonstrated among six bovine strains, all of serological type 1 ? subtype.

8. Mice immunised against a bovine strain of serological type 3a showed significant protection against test doses of two bovine strains of serological type 1 ? subtype, and against test doses of two serologically different human strains.

### CONCLUSIONS

1. Mice and guinea-pigs can be immunised against an intraperitoneal test dose of group-B streptococci of human or bovine origin by a series of intraperitoneal inoculations with living or formolised whole cultures of the homologous strain: this immunity is frequently demonstrable up to at least two months after the last immunising inoculation.

2. The method of mouse immunisation described may be used to examine immunological relationships among different strains of group-B streptococci.

I wish to thank Mr S. J. Edwards for five of the bovine strains, Mr J. I. Taylor for the two other bovine strains and the two human strains, and Mr P. Stuart, working under the direction of Dr A. W. Stableforth, for examining all these strains serologically. This work was carried out under the general direction of Dr W. S. Gordon.

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# THE EFFECT OF CASTRATION ON THE ACTION OF SOME BARBITURATES

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LATE in 1937, one of us noticed that spayed adult rats are more susceptible to the quick-acting barbiturate, pentobarbital sodium, than normal female rats (Cameron, 1938-39). A programme for the investigation of this phenomenon had to be put aside during the pre-war and war periods for more timely occupations, but recently we have returned to the problem and this paper describes the results of our experiments.

## *Methods*

Forty fully grown healthy male Wistar rats, about 200 g. body weight, were divided into two groups of twenty and kept ten in a cage in the same animal house, where temperature and humidity were uniform throughout the experimental period. A generous supply of the Rowett Institute rat cubes (Thomson, 1936) was provided daily for each animal, with unlimited water. Shortly before and at intervals after castration, the response to pentobarbital sodium and on one occasion also to barbital sodium was estimated in terms of the sleeping time. This method was used by Cameron and de Saram (1939) in a study of liver damage and barbiturate response, sleeping time being assessed as the interval in minutes between complete relaxation of all limbs and return of the righting reflex after intraperitoneal injection of 40 mg. per kg. body weight of pentobarbital sodium. There is a fairly wide range of variation of sleeping time in a large group of rats, but the mean values for groups are comparable, although as we show later on in this paper, there may be a steady increase in the mean as the animals age. For the barbital test we injected 150 mg. per kg. body weight intraperitoneally. During all tests care was taken to prevent heat loss, which may be severe with barbiturate anaesthesia.

Twenty rats were castrated under ether anaesthesia through a mid-line sub-umbilical incision, the spermatic cords and adherent tissues being ligatured with cotton thread and the abdominal wound closed with two layers of cotton sutures. Very little blood was lost and the animals made an uninterrupted recovery. Stitch abscesses seldom developed in castrates but were quite common in the controls. Laparotomy only was performed on the twenty control rats, the operative procedures being identical with those adopted in the castrated group except for the removal of the testes. No tests with barbiturates were instituted until healing was complete in all animals.

The experiment extended over 225 days (from March to November 1947). Twenty-four hours before the animals were killed they were given a subcutaneous injection of 0.5 c.c. of chloroform per kg. body weight so as to determine whether

castration alters the susceptibility of the rat to this liver poison. One animal in each of the two groups died within 24 hours, the remainder appearing ill. The adrenals, thyroids, pituitary and liver were carefully dissected out from each rat and weighed. Paraffin sections were prepared from representative portions of the liver and stained with Ehrlich's acid hæmatoxylin and eosin and Weigert's iron hæmatoxylin and van Gieson.

### Results

Tables I and II summarise the results of our experiments. Before castration the mean sleeping times for the two groups of 20 rats were  $64 \pm 5$  minutes and  $56 \pm 4$  minutes, a difference which is not statistically significant. With castration, the mean increased to  $127 \pm 6$  minutes after 63-67 days and to  $172 \pm 8$  minutes after 102-106 days. At this stage the standard dose of pentobarbital sodium exerted toxic effects and 4 castrates died. At 221 days after castration the mean sleeping time for the 16 survivors was  $157 \pm 9$  minutes, but 8 of the rats had gone through an interval of treatment with testosterone propionate which had lowered their response to pentobarbital sodium and this no doubt accounts for the fall in the mean for the group. These increases are statistically significant, so that we may conclude that castration lowers the tolerance of the male rat to the barbiturate pentobarbital sodium. This deduction is supported by consideration of the responses in the control rats, all of which were much less than those exhibited by the castrated animals. Statistical analysis bears out this impression.

Table I also shows an interesting variation in the mean sleeping times of the control series, with a significant increase from  $56 \pm 4$  to

TABLE I

*Response to pentobarbital sodium of castrated rats. Mean body weights and sleeping times*

Time Interval	Castrates			Controls		
	No. of rats	Body wt. (g.)	Sleeping time (mins.)	No. of rats	Body wt. (g.)	Sleeping time (mins.)
3 days before castration (March)	20	197	$64 \pm 5$	20	216	$56 \pm 4$
63-67 days after castration (June)	20	220	$127 \pm 6$	20	236	$68 \pm 5$
102-106 days after castration (August)	20	240	$172 \pm 8$ (4 died)	20	257	$77 \pm 5$
221 days after castration (November)	16	250	$157 \pm 9$ *	20	254	$97 \pm 4$

\* Includes the 8 rats treated with testosterone over 48 days; treatment ceased 59 days before this response to pentobarbital sodium was estimated.

$97 \pm 4$  over 224 days. We do not know the explanation of this lowered tolerance but think it is most likely an ageing effect. It stands in marked contrast to the partial increase in tolerance to barbiturates known to be acquired by some species (Hoff and Kauders, 1926; Fitch, 1930; Seevers and Tatum, 1931; Tatum, 1939).

Further evidence that the castration effect is a real one comes from the group of castrates treated with testosterone propionate (table II). Although the number of animals was rather small the

TABLE II

*Response to pentobarbital sodium of castrated rats treated with testosterone propionate (0.2 c.c. subcutaneously for 12 injections over 48 days). Mean body weights and sleeping times*

Time Interval	Castrates					
	Testosterone group			No testosterone		
	No. of rats	Body wt (g)	Sleeping time (mins)	No. of rats	Body wt (g)	Sleeping time (mins)
3 days before castration . . .	8	185	72 $\pm$ 7	8	209	57 $\pm$ 1
67 days after castration . . .	8	209	121 $\pm$ 14	8	236	129 $\pm$ 6
106 days after castration . . .	8	224	179 $\pm$ 11	8	257	165 $\pm$ 11
134 days after castration . . .	8	256	119 $\pm$ 14	8	235	162 $\pm$ 10
	(after 6 injections of testosterone)					
170 days after castration . . .	8	263	96 $\pm$ 7	8	255	127 $\pm$ 5
	(after 12 injections of testosterone)					
221 days after castration . . .	8	251	145 $\pm$ 13	8	248	169 $\pm$ 8

results are so consistent that we have no hesitation in accepting the outcome of this experiment. The first group of 8 rats showed a mean sleeping time of 72  $\pm$  7 minutes before castration, which increased to 179  $\pm$  11 minutes 106 days after castration. Six injections of 0.2 c.c. testosterone propionate subcutaneously given over a period of 28 days reduced the mean sleeping time of this group to 119  $\pm$  14 minutes. After a further 6 injections the mean sleeping time fell to 96  $\pm$  7 minutes. In the second group of 8 castrated rats the sleeping time remained high in the absence of testosterone administration, although there was a slight decline at one time. Statistical analysis shows highly significant differences between the pre-castration and post-castration sleeping times throughout the experimental period in this group. Clearly, replacement therapy with testosterone exerted a beneficial effect upon barbiturate tolerance. When testosterone was discontinued the mean sleeping time again lengthened, so that the sex hormone influence appears to be a reversible one, at any rate under the conditions with which we have been concerned.

In striking contrast was the reaction to barbital sodium at a time when the pentobarbital sodium tolerance had greatly decreased. Intraperitoneal injection of 150 mg. per kg. of barbital sodium induced a mean sleeping time of 84  $\pm$  13 minutes in 20 castrated rats; under similar conditions the 20 controls slept 96  $\pm$  15 minutes. No significant difference thus exists between the means for the two groups, the ratio of the difference to the standard error of the difference being

much less than two. The significance of this observation will be commented on later.

Finally, we were unable to determine any difference in the extent and severity of the injury induced by chloroform in the two groups of animals.

### Discussion

Our investigation establishes a relationship in male rats between testicular function and tolerance to the barbiturate pentobarbital sodium, for castration leads to a significant increase in the sleeping time induced by a standard dose of pentobarbital sodium, which can be greatly inhibited by replacement therapy with testosterone propionate. No such relation exists in the case of barbital sodium.

Other workers have come to a similar conclusion from investigations in which several barbiturates were employed. Holck *et al.* (1937 *a* and *b*, 1942), unbeknown to us, found a lowered tolerance to pentobarbital, evipal and pernoston in castrated rats but not to barbital. The action of testosterone esters in raising the resistance of female and male castrates was also demonstrated by Holck *et al.* (*loc. cit.*), Störtebecker (1939) and Kinsey (1940), but no explanation of the phenomenon has been offered by these workers. We wish to put forward some suggestions as to the meaning of these observations, with a hypothesis linking the function of the liver with that of the sex glands.

The depressant barbiturates may be conveniently grouped as (1) long acting, *e.g.* barbital and phenobarbital, (2) intermediate, *e.g.* neonal, dial, nostal, (3) short acting, *e.g.* amytal, pentobarbital and phanodorm, (4) ultra-short acting, *e.g.* evipal and pentothal (Werner *et al.*, 1937).

Generally speaking, the long-acting compounds are eliminated from the body almost entirely by the kidneys, the liver playing a minor role. This certainly appears to be the case with barbital (Pratt *et al.*, 1932; Pratt, 1933; Cameron and de Saram, 1939). The short-acting barbiturates are mainly destroyed by the liver, although small amounts may be eliminated by the kidneys or dealt with by other tissues (Pratt, 1933; Cushny, 1936; Cameron and de Saram, 1939; Goodman and Gilman, 1941; Richards and Appel, 1941). Pentobarbital sodium may thus be used as a means of assessing one of the detoxication mechanisms in the liver of experimental animals, provided there is no reason to suspect altered susceptibility of the central nervous system to the barbiturate. Likewise in the absence of renal insufficiency barbital may serve as an indicator of nervous sensitivity to barbiturates. We have made these assumptions in the present investigation and conclude that, because the sleeping time induced by a standard dose of pentobarbital sodium progressively increases and the barbital sleeping time does not, there is a disturbance of liver function associated in some manner

with castration. Moreover, this impairment can be repaired, at least in part, by restoration of one of the missing sex hormones—testosterone, but promptly recurs when the hormone is discontinued. We would therefore suggest that in the normal animal detoxication of pentobarbital sodium is to some extent under the control of the sex hormones, for we now have evidence (Cameron, 1938-39) that both male and female rats respond to gonadectomy in similar fashion so far as their reaction to pentobarbital sodium is concerned.

The idea that a functional relationship exists between the liver and the sex organs is not a new one. In recent years a steady accumulation of facts supports the view that the liver inactivates various sex hormones.

1. Oestrogens are said to be inactivated after passage through a heart-lung-liver perfusion system but not a heart-lung system (Israel *et al.* 1937).  $\alpha$  Oestradiol is removed rapidly from rat-liver perfusates (Schiller and Pincus, 1943; Heller, 1944; Schiller, 1945). It is true that Cantarow *et al.* (1943) maintain that exogenous and endogenous oestrogens are not destroyed in the liver but are excreted in the bile, but this opinion has been challenged by Schiller and Pincus, who point out that Cantarow *et al.* did not take into account the inter-conversion of oestrogens.

2. Oestrogens are inactivated *in vitro* by liver slices or pulp (Zondek, 1934; Heller, 1940; Twombly and Taylor, 1942; Zondek *et al.* 1943).

3. Pellets of oestrone and oestradiol benzoate are less potent when they are implanted into the spleen (Biskind and Mark, 1939; Biskind, 1941; Kochakian *et al.* 1944) than when inserted into somatic tissues. Seventeen times as much diethylstilboestrol is required for the production of vaginal oestrus in 50 per cent. of spayed rats when the injection is given into the spleen *in situ* as when the injection is given into a transplanted spleen (Segaloff, 1943, 1944). In other words, when the hormone has to pass through the liver before reaching the systemic circulation, it loses much of its potency.

4. Endogenous oestrogens are inactivated when the ovaries are implanted intraperitoneally (Golden and Sevringhaus, 1938).

5. Uteri of young female rats show stimulation by endogenous oestrogens after the liver has been damaged by carbon tetrachloride or alcohol (Talbot, 1939). Partial hepatectomy interferes with the conversion of oestrone to oestriol, much of it being excreted in the urine unchanged (Schiller and Pincus, 1944).

6. Whole liver extract is said to reduce the mortality of rats receiving toxic doses of diethylstilboestrol (Forbes and Evans, 1943).

Such experiments suggest very strongly that certain sex hormones are excreted or converted into inactive forms by the liver. If the hepatic tissue is damaged by poisons or reduced in amount by operative procedures, enhanced activity of the hormones follows, suggesting that they are able to pass the liver barrier without interference. In



this way the hepatic cells exert an important control on the internal secretory activity of the sex glands.

There is some reason to suspect that the liver may be influenced in another fashion by the sex glands. Victor *et al.* (1936) have described variations in liver cell respiration throughout the sexual cycle of rats. The rate of oxygen consumption of liver slices *in vitro* is highest at oestrus, lowest in spayed animals and intermediate between these levels during dioestrus. Castrated rats are said to show fewer binucleate cells in their livers than normal animals and the administration of oestrogens and progesterone appears to increase the number of such cells (Allan, 1944). The liver of castrated male rats along with other organs shows some atrophy (Korenchevsky and Dennison, 1934) and its lobules decrease in size (Hall and Korenchevsky, 1938), especially when castration is performed before sexual maturity has been reached. These facts hint at a diminution of metabolic activity in the liver cells after castration and perhaps detoxifying mechanisms become impaired because of this. Such a hypothesis would explain the altered barbiturate response, but obviously the matter needs to be put to experimental test and this we hope to do shortly.

We have now reached the interesting position of accepting a function in the liver whereby it detoxicates sex hormones and barbiturates, and of demonstrating that absence of certain sex hormones damp down further the activity of the liver mechanism which deals with quick-acting barbiturates. Can it be that these hormones in being disposed of by the liver cells are used as part of a detoxication process, either intimately as co-enzymes or indirectly through an action on the liver cell metabolism? Is this relationship a direct one or mediated through some other internal secretion? Atrophy of the thyroid glands and hypertrophy of the adrenals and pituitary are usual accompaniments of castration (Korenchevsky, 1930 (lit.): we can confirm this from our experiments). Such speculations must not be pushed too far in the absence of relevant facts, but it seems to us that here is a profitable field for further investigation.

### *Summary*

Castrated male rats develop a lowered tolerance for quick-acting barbiturates such as pentobarbital sodium but not for barbital sodium, a slow-acting compound. Replacement of one of the missing hormones, testosterone, is followed by improved tolerance. Ageing of normal rats is accompanied by progressive, though not profound, lowering of tolerance. It is suggested that the altered barbiturate response indicates disturbance, probably reversible, of a functional relationship between the sex glands and the liver. Already existing evidence that the liver inactivates sex hormones is discussed, and the hypothesis is advanced that such hormones are utilised as adjuvants or co-enzymes in liver detoxication.

We are indebted to Dr E. W. Horning for advice about the use of testosterone and for a generous gift of that compound.

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CLOTTING OF HUMAN CITRATED PLASMA BY  
GRAM-NEGATIVE ORGANISMS

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COAGULATION of human plasma is now accepted as the most constant biochemical criterion in the recognition of a pyogenic *Staphylococcus aureus* (see Much, 1908; and for recent review Blair, 1939). It is also generally inferred that when plasma is clotted as the result of bacterial action the organism responsible is *Staph. aureus*. The ability of other organisms to clot human plasma has been but little investigated. Loeb (1903-04), who originally studied the action of various organisms on plasma, found that *Bacterium coli* and *Pseudomonas pyocyanea*, one strain of each, clotted goose plasma; and Fisher (1936) noted that two strains of *Bacillus subtilis* and one of *Ps. pyocyanea* were capable of clotting rabbit but not human plasma.

In the course of diagnostic routine work an attempt was made to use for coagulase tests the mixed surface growth of agar cultures instead of the isolated strain of *Staphylococcus*. On several occasions clotting occurred with a mixed culture from which only various Gram-negative organisms could be isolated; one of these organisms, a member of the paracolon group, was found to be responsible for the clotting. This observation seemed to challenge the specificity of the coagulase test, and the present work was undertaken to discover which organisms apart from pyogenic *Staph. aureus* were capable of clotting plasma and what was the mechanism involved.

## EXPERIMENTAL

Thirteen strains of Gram-negative organisms were used (table I). Human plasma (blood bank) containing 0.4-0.7 per cent. sodium citrate and 1 per cent. glucose was employed throughout. Each batch of plasma was tested for clotting on recalcification by adding one drop of 5 per cent. calcium chloride to 0.5 c.c. of plasma diluted 1 in 2; only batches which clotted were used.

In preliminary experiments 0.5 c.c. of human citrated plasma diluted 1 in 5 with saline was inoculated with two drops of 24-hour broth cultures of various Gram-negative organisms and incubated

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\* Work carried out by N. S. Conway during the tenure of a McCunn Research Scholarship.

at 37° C. The results were read at intervals: within 18 hours soft clot had formed with 11 of the 13 strains. In order to find the maximum dilution at which plasma could be made to clot by these organisms, doubling dilutions of plasma in saline ranging from 1 in 1 to 1 in 16 were set up, inoculated, and incubated for 18 hours; firm clot formed up to and including the 1 in 4 dilution, soft clot at 1 in 8 and no clot at 1 in 16. Accordingly, all subsequent experiments were carried out with plasma diluted 1 in 2.

Since it appeared possible that an enzyme analogous to that produced by *Staph. aureus* (staphylocoagulase) was responsible for the clotting, the following experiment was carried out. The organisms were grown at 37° C. for 18-24 hours in a mixture of meat-extract broth (2 parts) and plasma (1 part). The resulting clot was disintegrated by shaking and the culture passed through a Seitz filter (grade S.B., Ford's "Sterimat") which was thoroughly washed before filtration by passing saline through it in order to remove soluble calcium compounds. The filtrates were found to lack clotting power. The possibility that acid produced by the organisms formed the clot was next investigated; however, the organisms tested caused a rise in pH which *per se* does not clot controls.

The question then arose whether clotting of citrated plasma might be due to bacterial breakdown of citrate with subsequent liberation of calcium ions, since various Gram-negative organisms are known to utilise citrate as a source of carbon. Whether or not a particular organism can utilise citrate is usually determined by the presence or absence of its growth in Koser's medium (Koser, 1923) in which nitrogen is present in inorganic form (ammonium phosphate) and carbon is supplied by sodium citrate. Accordingly, organisms which in the preliminary experiments were found to clot plasma, were inoculated into Koser's medium; only some were found to be Koser-positive (table I). This at first seemed to invalidate the hypothesis that clotting was due to utilisation of citrate and liberation of calcium. It has been shown, however, (Lominski *et al.*, 1947) that Koser-negative organisms can utilise citrate in the presence of a source of nitrogen other than ammonium phosphate, such as asparagine or peptone; it seemed probable that in plasma the proteins might in an analogous way provide a suitable source of nitrogen. Since, however, growth in plasma will occur regardless of citrate utilisation, the turbidity of the fluid due to bacterial multiplication could not, as in Koser's medium, be used as an index of citrate breakdown, and chemical estimation of the citrate became necessary. For this purpose plasma was diluted in bulk in order to avoid variation in the citrate content from flask to flask, and 10 c.c. volumes were distributed under sterile conditions into Erlenmeyer flasks: these were inoculated and incubated at 37° C. for 18 hours. The clot was disintegrated by shaking and the citrate content of the total unfiltered fluid determined by the Hunter and Leloir (1945) modification of the method of Pucher, Sherman and

Vickery (1936). A number of uninoculated flasks were also incubated and served as controls. The results indicate that five strains which do not utilise citrate in Koser's medium are capable of doing so in presence of plasma proteins, and that clotting runs parallel with the utilisation of citrate in plasma (see table I). The average citrate

TABLE I

*Showing the correlation between clotting and the utilisation of citrate by Gram-negative organisms*

Organisms	Clotting of plasma	Citrate utilisation in	
		Koser's medium	plasma
<i>Bact. coli</i> (intermediate strain) .	+	+	+
" " var. <i>communis</i> . .	+	—	+
" " " " . .	+	—	+
<i>Bact. coli</i> var. <i>communior</i> . .	+	—	+
" " " " . .	+	—	+
<i>Bact. aerogenes</i> . . . .	+	+	+
<i>Bact. cloacæ</i> . . . .	+	+	+
" " (intermediate strain) .	—	—	—
<i>Paracolon bacillus</i> . . . .	+	+	+
" " . . . .	+	+	+
" " . . . .	+	+	+
" " . . . .	+	—	+
" " . . . .	—	—	—

content of plasma after incubation in flasks inoculated with clotting or non-clotting organisms and of uninoculated controls is shown in table II; it is clear that clotting occurs when the concentration of

TABLE II

*Showing the relation between clot formation and citrate concentration*

Plasma (1 in 2)	Average citrate content (mg./100 c.c.)
Uninoculated control . . . .	210
Inoculated with clotting organisms . .	2
" " non-clotting organisms . .	200

citrate falls below the level required for the binding of free calcium ions (Harrison, 1947). If the ability of organisms to clot citrated plasma depends solely on their power to utilise citrate, clotting should not occur under the following conditions; namely, when the plasma contains initially more citrate than the organism is capable of utilising,

in a plasma whose calcium is bound by a non-metabolisable compound, e.g. oxalate, and in a plasma where an element of the blood-clotting mechanism is either lacking or inhibited. Accordingly the three following experiments were carried out. (i) Concentrations of citrate ranging from 0.25 to 5 per cent. were prepared in plasma diluted 1 in 2 with saline and inoculated with a strain previously shown to clot the routine citrated plasma. After 18 hours' incubation, clot formed in the tubes up to and including 1 per cent. but never above this concentration, although there was good growth. (ii) The organisms were grown in oxalated plasma; no clot was formed. (iii) Use was then made of plasma passed through a Seitz pad (such plasma does not clot on recalcification but coagulates on addition of thrombin, showing that fibrinogen is present) and plasma in which the thrombin mechanism was inactivated by heparin (2.5 Toronto units per c.c.). In both cases the plasma failed to clot when inoculated with the organism under investigation; this was not due to inhibition in the breakdown of citrate, since chemical estimation showed that the organisms had used practically all the citrate present.

### DISCUSSION

Experimental evidence has been produced which shows that certain Gram-negative organisms are capable of clotting human citrated plasma and that the phenomenon is due to the breakdown of citrate by these organisms; the subsequent liberation of calcium ions operates the normal mechanism of blood clotting. Therefore they fail to clot citrated plasma whose blood-clotting agents have been removed by filtration or inactivated by heparin. These organisms also fail to clot plasma when citrate is in excess or when calcium is bound by a non-metabolisable salt (oxalate). No coagulating enzyme appears to be produced by Gram-negative organisms. Lastly, clotting by citrate-utilising bacteria can occur only in relatively undiluted plasma (1 in 8), since breakdown of citrate and subsequent liberation of calcium ions will remain ineffective in a plasma whose blood-clotting elements are too dilute to act.

Such a mechanism is basically different from that of plasma clotting by *Staph. aureus*; here coagulation is due to the production of a clotting enzyme by the organism and not to the progressive destruction of the plasma anti-coagulant. Clotting by citrate-utilising bacteria, depending as it does on citrate destruction, is determined by the initial amount of citrate present and by the rate of growth of the organisms; this explains the long period necessary, 18 hours or more, for clot formation. In contrast to this, clotting by staphylocoagulase depends on the concentration of the enzyme present and accordingly varies considerably in speed; if pre-formed enzyme is present in high concentration, addition of the culture to plasma may cause almost instantaneous clotting. Also, in contrast to Gram-

negative organisms staphylocoagulase clots plasma irrespective of whether citrate, oxalate or heparin is used as anticoagulant. Finally, clotting by staphylocoagulase occurs even if the plasma is diluted 1 in 100, being limited mainly by the amount of fibrinogen present.

These findings indicate that clotting of plasma by bacteria may be due either to the production of a coagulating enzyme or, as in the present instance, to the breakdown of the anti-coagulant—citrate. Accordingly coagulase tests, which aim at revealing the presence of *Staph. aureus* by demonstrating the action of its clotting enzyme, should always be performed with heparinised plasma. If, as is usual, only citrated plasma is available, heparin should be added. Further, since citrate is the customary anti-coagulant used in the storage of blood for transfusion purposes and since some Gram-negative organisms grow well at low temperatures, it is possible that clotting in bottles of plasma is occasionally due to contamination with these organisms; therefore the contents of all bottles in which a clot is present must be discarded.

### SUMMARY

Clotting of plasma by a variety of Gram-negative organisms has been described.

The mechanism of this clotting depends on the breakdown of citrate, the subsequent liberation of Ca ions throwing into operation the normal blood-clotting system.

Since only citrated plasma is clotted, heparin should always be added to plasma which is to be used for the coagulase test.

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# ANTHRAX IN A LABORATORY WORKER, WITH OBSERVATIONS ON THE POSSIBLE SOURCE OF INFECTION

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DESPITE the precautions normally taken to prevent infection or contamination with *Bacillus anthracis*, laboratory workers sometimes become infected. In 1947 one of my students became infected during or after a practical class in which he handled fixed and stained anthrax smears. A typical lesion developed on his chin at the site of a cut made during shaving, and virulent anthrax bacilli were isolated from it. Fortunately the infection was mild and the student recovered after treatment in hospital.

From investigation of all likely sources it was concluded that the infection must have been acquired from handling either fixed films made from an anthrax culture or blood smears prepared from a guinea-pig which had died of anthrax. The class during which the student handled these preparations met about 12 days before the infection appeared. This would be rather a long period of incubation and it seemed probable that he acquired the infection during re-examination of the slides rather than during the practical class itself. The culture films were made from 24-hour anthrax cultures and were fixed by heat before they were handed to the students for staining. The blood smears from the guinea-pigs were also handed to the students after fixation.

Since this accident suggested that despite fixation and staining one or other of the preparations contained viable organisms it was decided to collect all anthrax slide preparations from the students in order to check this possibility. Each slide was rubbed with a moist swab and cultured. All slides made from the anthrax culture yielded growths of anthrax bacilli, whereas some of those prepared from the guinea-pig blood films were positive and others were negative. This suggested that the infection was more likely to have come from the anthrax culture film. In further investigations, anthrax culture films which had been made in the department over a period of a few years were examined for viability. From most of these preparations *B. anthracis* was cultivated. These results stimulated further investigations into preparations with anthrax organisms, and it is the purpose of this communication to report their results.

*Survival of anthrax spores on slides after various forms of treatment*

The literature on the resistance of *B. anthracis* spores to dry heat, reviewed by Murray (1931), is still controversial. Most workers claim that hot air kills spores of *B. anthracis* in 3 hours at 140° C. and in 1½ hours at 160° C. Murray himself found that at 135° C. the time ranged between 5 and 10 minutes, at 125° C. it was 25 minutes and at 120° C. it ranged between 40 and 45 minutes. Recently Oag (1940), using an electric furnace as a source of dry heat, found that a temperature of 400° C. kills the spores of *B. anthracis* in 20-30 seconds and at 120° C. in an hour. According to Oag (p. 141), "The time necessary to fix a film of spores is very much less at any given temperature than that required to kill the organisms".

## EXPERIMENTAL OBSERVATIONS

Two strains of *B. anthracis* were used: the strain "G" isolated from the above student—and "L" an old laboratory strain.

*I. The time needed to kill spores of B. anthracis on slides*

Films made on slides were heated in the upper part of a Bunsen flame for periods ranging from 1 to 7 seconds. The films were then left on the bench for a short time and, when cool, were rubbed with swabs soaked in sterile broth; these in turn were smeared on blood-agar plates which were incubated at 37° C. for 24 hours. The films heated up to 5 seconds gave positive cultures, whereas those heated for longer than 5 seconds were negative. The films heated for more than 5 seconds were usually burnt and of little value for microscopical examination.

*II. The effect of dyes and methods of staining on the viability of spores of B. anthracis on slides*

Smears from sporulating 3-day cultures on agar fixed for about three seconds over the Bunsen flame were stained by the following methods:—Gram's method, Ziehl-Neelsen's method and its modification for staining spores, Muir's method and Hiss's method for capsules and Schaeffer and Fulton's (1933) malachite-green method for spores. The following simple stains were also employed:—crystal violet 0.5 per cent.; gentian violet 1 per cent.; carbol fuchsin 1 per cent. in 5 per cent. carbolic acid; and methylene blue in 1 per cent. aqueous solution. After being stained, each preparation was rubbed with a swab moistened in sterile broth and the swab was smeared on a blood-agar plate.

After fixation and staining, all these slides contained viable spores, even when the spores themselves were stained.

*III. The effect of mercuric chloride and potassium permanganate on the spores of B. anthracis on slides*

Films containing sporulating *B. anthracis* were immersed for periods ranging from ½ to 60 minutes in a 1:1000 solution of mercuric

chloride. Each film was then rinsed under running water, dried, and washed off with a moistened swab, which was smeared on a blood-agar plate.

Similar experiments were carried out with saturated and with 4 per cent. solutions of potassium permanganate.

Growth was not obtained from films immersed in 1 : 1000 mercuric chloride for 5 minutes or longer. Those treated with potassium permanganate were negative after immersion for 5 minutes in the saturated solution or 15 minutes in the 4 per cent. solution. The films treated with potassium permanganate were so discoloured as to make them unsuitable for staining, but the preparations immersed in mercuric chloride were fixed and stained well.

### *Spores in the freshly opened carcass*

Hitherto it has been assumed that greater risk was attached to the handling of preparations made from sporing cultures than of smears prepared from the freshly opened carcass of an animal which had died from anthrax. This assumption is based on the widely accepted view that *B. anthracis* does not sporulate in the unopened carcass, but the point that not all anthrax spores are changed into vegetative forms in the animal body when a culture is inoculated is quite often overlooked. Howie and Cruickshank (1947) found viable anthrax spores in the spleen of mice killed 3 months after inoculation of *B. anthracis* spores. In order to inoculate bacilli without spores Howie (personal communication) adopted the plan of injecting a 4-hour culture and then making 3 or 4 serial passages direct from the heart blood of infected mice. With such heart blood as a source of asporogenous bacilli he never found heat-resistant forms in the carcass of an injected animal but observed that vegetative forms from the carcass developed heat-resistant spores if the blood or organ was prevented from drying and kept at a temperature above 18° C. In my experiments I sought to produce an asporogenous anthrax culture for animal inoculation without having recourse to repeated passages through animals by the following method.

*B. anthracis* was subcultured several times at 37° C. under 50 per cent. carbon dioxide on blood-agar plates. Microscopic examination of stained smears of the fourth subculture showed that, as far as one could determine by this method, it was asporogenous. Ten guinea-pigs were inoculated subcutaneously with 0.5 c.c. of a suspension from such a culture. All died within 24 hours, whereas guinea-pigs injected with a suspension of a sporulating culture do not usually die until 2-3 days later.

Immediately after the carcass was opened the spleen and heart were transferred to a sterile Petri dish and smears from blood and splenic pulp were prepared with a minimum of delay. The smears were fixed by heat for 5 seconds and stained with a one per cent. solution of aqueous methylene blue. After microscopic examination the slides were rubbed with moistened swabs and

cultivated on blood-agar plates. The remainder of the spleen and heart were each separately suspended in saline and heated at 90° C. in an oil-bath for 5 minutes. When cool they were cultured on blood-agar plates.

The results of these investigations were as follows :—

- (1) A careful and prolonged microscopical examination of freshly prepared smears from the carcass showed the presence of spores.
- (2) The fixed and stained smears from the heart blood and spleen gave cultures on blood agar.
- (3) Cultures were also obtained from the heated spleen and heart tissue.

It seems likely that a microscopic examination alone cannot be relied upon to determine whether a culture contains spores or not. Although microscopic examination and the rapid death of the guinea-pigs seemed to suggest that the culture was asporogenous some spores may have been present in the inoculum, which was not examined after heating. The point of practical importance is that one cannot assume that freshly prepared and rapidly fixed blood films from guinea-pigs which have died of anthrax after injection of cultures are necessarily free from spores which were present in the inoculum.

*Presence of viable anthrax spores in condensation droplets from heated suspensions of anthrax spores*

According to the recent studies of Stein and Rogers (1946) it would appear that viable anthrax spores may escape into the atmosphere during the heating of anthrax suspensions in open containers.

To examine this question anthrax spore suspensions were made by emulsifying 3-day cultures in 2 c.c. of saline in 6×1 in. pyrex test-tubes with cotton-wool plugs. To avoid contaminating the upper portions of the tubes with spores, the suspensions were made with the greatest possible care. To make quite certain that the upper part of the tube in the region of the cotton-wool plug was absolutely free from anthrax spores, controls were made by swabbing this part of the tube and making cultures. The suspension was then heated in an oil-bath at 90-95° C. for 1-2 minutes. During heating, vapour from the spore suspension formed condensation droplets on the neck of the tube. These condensation droplets were then swabbed and smeared on blood-agar plates. The cotton plugs were also smeared on the same plates, which were incubated for 24-48 hours. All swabs from both condensation droplets and cotton-wool plugs proved on cultural examination to be anthrax-positive, whereas control swabs made from the necks of the tubes before steaming were negative. Since it seems unlikely that anthrax spores could be deposited in this

situation from water vapour, their presence on the cotton-wool plugs was probably due to trapping by the wool of minute droplets of fluid thrown up by the breaking of the bubbles.

### SUMMARY

1. Spores of *B. anthracis* in films on slides may withstand the temperature of the Bunsen flame for 5-6 seconds.

2. None of the common stains and methods of staining used in bacteriological technique destroyed all spores in films on glass slides.

3. Mercuric chloride solution (1:1000) killed the spores of *B. anthracis* smeared on slides after these had been immersed for 5 minutes. Spores were also destroyed by a saturated solution of potassium permanganate after 5 minutes' exposure, and by a 4 per cent. solution after 15 minutes' exposure. Potassium permanganate, however, rendered the slide unsuitable for further staining.

4. Guinea-pigs infected with cultures of *B. anthracis*, which may have contained spores, invariably showed the presence of spores in the recently opened carcass.

5. Evidence was obtained that viable spores may escape into the atmosphere if spore suspensions of *B. anthracis* in saline are heated in open containers.

I wish to express my thanks to Professor A. W. Downie and Dr G. O. Davies for their helpful criticism of the paper and to Mr L. Kelley for his technical assistance.

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# THE LINING OF HEALED BUT PERSISTENT ABSCESS CAVITIES IN THE LUNG WITH EPITHELIUM OF THE CILIATED COLUMNAR TYPE

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(PLATES XXXIII-XXXVI)

ABSCESS cavities in the lung, like similar lesions elsewhere, heal in one of two ways: by obliteration and scarring, or by epithelialisation. In most situations abscess cavities which become epithelialised acquire a lining of squamous epithelium. In the lung, however, abscess cavities which persist sometimes become lined with ciliated epithelium. Attention has been drawn to such lesions by Berard *et al.* (1946) and by Sellors *et al.* (1946). In the latter paper 27 cases were described in which a suppurative process affected different parts of the lung in succession. Three cases were treated by pneumonectomy, and in two of these the specimen contained cavernous spaces lined by ciliated columnar epithelium. There was little doubt about the acquired character of the lesions, because they were observed radiologically to arise in parts of the lung which had been previously normal. The excised lung, in one case with a four years' history, contained cavities of different types. Those which developed in the early stages were lined with ciliated epithelium; they contained air, appeared to be undergoing ciliary drainage and were usually clean. Those which formed later, when the disease was more advanced, were lined with squamous epithelium and contained stale pus, while the most recent were lined with granulation tissue and contained a purulent slough. It would appear that in cases of this kind ciliated epithelium forms when conditions are favourable for healing, squamous epithelium when they are unfavourable. Simple surgical drainage could not close these cavities, and the French workers were concerned with the possible prevention of epithelialisation.

In spite of these reports the process of ciliary epithelialisation is not well known, and in the present paper the thesis is restated and five more cases are described. The current pulmonary symptoms in these cases were those of bronchiectasis, and included persistent cough with excessive sputum which was usually non-fœtid. In four cases, however, the history began with symptoms suggestive of lung



abscess or pneumonia. This illness was characterised by fever, pleuritic pain and cough, with sputum which in most cases was foul. Sometimes this attack was repeated. In one case the history was unsatisfactory.

The appearances of the cavities in these (and the previous) cases are shown diagrammatically in fig. 1. Some large cavities (L1) had large bronchi opening directly into them. Other large cavities had only small bronchial connections. Some of these formed extensive clefts deep to the surface (L2) or alongside the large bronchi, but communicating with them only by small ostia. Most small cavities (S) had only narrow communications, whether connected with large or small bronchi. Some, however, appeared to have communicated widely, and to have been responsible for irregular dilatation of some bronchi (P). Some small cavities (T), situated in the angle between bronchi, made these intercommunicate immediately beyond their origin. Many cavities appeared to have formed by confluence of lesions which were once discrete. Some cavities extended across septa and fissures, and communicated with bronchi of separate sectors or lobes. Many cavities were bridged by trabeculae (fig. 2).

These appearances were readily explicable on the basis of an ulcerative process, and even though the lesions had now a lining of ciliated epithelium, this was their probable origin. These appearances may of course be seen in congenital abnormalities, but such lesions require an additional ulcerative factor to explain them. Several of the cases of intra-lobar sequestration described by the author (Pryce, 1946; Pryce, Sellors and Blair, 1947-48) were in fact complicated in this way. The heterodox view which regards the mesenchyme as playing the major role in modelling the embryonic bronchial tree, which might be invoked to explain the present lesions on a congenital basis, cannot be admitted.

The only difficulty in accepting the ulcerative theory appears to be the assumption, which is tacitly but widely made, that ciliated columnar epithelium is too highly differentiated to undergo reparative growth. But the proliferative capacity of ciliated bronchial epithelium has been experimentally demonstrated. Thus when small pieces of rabbit lung are placed in tissue culture media the ciliated epithelium grows over the cut surface of removal (Robb-Smith, 1936). In some instances the ciliary waves acting rhythmically in one direction are capable of rotating the whole fragment. Again when small incisions are made into the lungs of cats the cut bronchi sprout into the healing wound and even form new aerated lung tissue (Montgomery, 1943-44). The epithelium, at first cuboidal, later becomes ciliated, unless infection occurs, when it becomes squamous. Important also is an observation by Willis (personal communication) of the ciliary epithelialisation of a hydatid cyst after the evacuation of its contents into a bronchus.

In most places the ciliated epithelium lining the cavities is superimposed on scar tissue devoid of muscle, cartilage or glands (fig. 3), but naturally these structures are present where parts of communicating

EPITHELIAL LINING OF LUNG CAVITIES



FIG. 2.—Larger compartment of cavity in lower lobe in case 1.

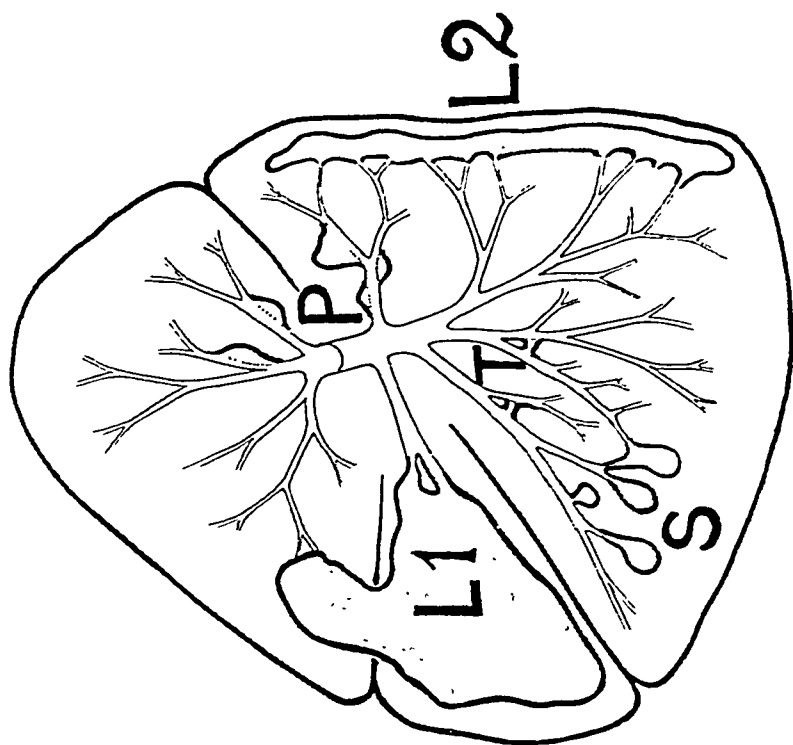


FIG. 1.—Diagrammatic representation of different types of cavity.  
 L1. Large cavity communicating with proximal bronchi.  
 L2. Cleft-like cavity near surface.  
 S. Small sacculus.  
 T. Small cavities between dividing bronchi causing intercommunication.  
 P. Dilatations of bronchi apparently due to parabronchial abscesses.



EPITHELIAL LINING OF LUNG CAVITIES



FIG. 3.—Wall of cavity in case 1 with ciliated columnar epithelium superimposed on scar tissue.  $\times 89$ .



FIG. 4.—Hypertrophied littoral epithelium in alveoli adjacent to fibrous wall of cavity in case 1. The epithelium, although in this instance columnar, is quite unlike the ciliated epithelium of the cavity.  $\times 80$ .



EPITHELIAL LINING OF LUNG CAVITIES



FIG. 6.—Wall of cavity in case 2, showing a trabecula of fibrosed lung covered by respiratory epithelium. 50.



FIG. 5.—Medial aspect of dissected specimen in case 2. Two branches of the middle lobe bronchus enter a large cavity which (on the other side of the specimen) extends into the upper lobe. The dorsal bronchus (which begins opposite the middle lobe bronchus) runs into two cavities, the upper of which shows a trabecula. These join a large cavity lateral to the basal bronchi.



EPITHELIAL LINING OF LUNG CAVITIES



FIG. 7.

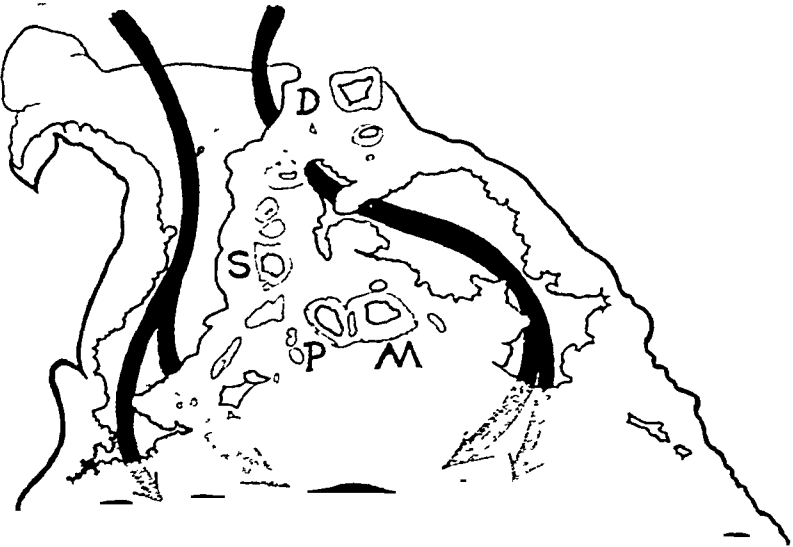


FIG. 8.

FIGS. 7 and 8.—Medial aspect and section of lower lobe in case 3, showing large cavity round periphery of lobe





bronchi are incorporated in the wall. The ciliated epithelium appears to grow from the mouths of bronchi which open into the cavity both distally and proximally. The process is apparently complex and dependent on many factors, some of which promote drainage and are purely mechanical. In this respect the size of the bronchial communication, the situation in the lung and the amount of secretion in the air passages are relevant. But the healing of basal cavities and of cavities with small communications indicates that more important factors exist. These combat infection and include the patient's resistance and treatment. It is probable that with modern chemotherapy healed cavities with ciliated epithelium will become more frequent, *i.e.* in lesions which would otherwise be fatal in the early stages or become lined with squamous epithelium in the later stages. Healed cavities with ciliated epithelium are not encountered in tuberculosis, actinomycosis or other conditions in which infection is not readily overcome, but future chemotherapy may change this too. Nutritional factors are also involved, *e.g.* vitamin A, deficiency of which causes squamous metaplasia. The process requires oxygen and depends on the supply of blood from the bronchial arteries, or, in the presence of adhesions, from anastomatic parietal arteries. The higher systemic pressure favours the flow of blood from these arteries, rather than from the pulmonary, to the new vessels of granulation tissue. The littoral epithelium of the pulmonary alveoli does not appear to participate in the lining of these cavities. The proliferation of this epithelium, which gives rise to the glandular appearance of certain forms of fibrosed lung, begins in isolated patches in the alveoli which abut on the fibrous framework of the lung (fig. 4). This epithelium may become columnar, but probably not ciliated. In the fibrosed lung near the cavities in two cases there was an excess of hypertrophied plain muscle. This, like the ciliated epithelium lining the cavities, appears to be a new formation. Its occurrence in certain cases of pulmonary fibrosis has been previously reported (*e.g.* Bowden, 1947).

The present lesions have a bearing on the problem of chronic bronchiectasis. In the proper connotation of the term (pathological dilatation of bronchi) this is a separate condition which may be due to distension beyond a block, traction by fibrosis, or infection of bronchi dilated by massive collapse; it may even be congenital. But the view is held that some cases of this heterogeneous group are due to ulceration (McNeil *et al.* 1929; Erb, 1933; Lisa and Rosenblatt, 1943; and many others). With this view, which according to Lisa and Rosenblatt was first put forward by Gairdner in 1851, the author would agree. Epithelialised abscess cavities such as would occasionally result from the healing of suppurative broncho-pneumonic foci would be indistinguishable from the lesions of certain cases of saccular bronchiectasis. But it is probable that the ulcerative process is more frequently secondary to a suppurative pneumonitis from without,

as in the present lesions, than to ulceration of the bronchial mucosa from within as described by the above authors.

**Case 1.** A soldier of 20 whose history began 15 months earlier with malaise, cough, foul sputum and hemoptysis. Although subsequent attacks occurred, his general condition remained good. He had some basal rales, however, and slight clubbing of the fingers, and X-ray examination revealed a large cavity at the base. Lobectomy was performed, and he returned to the forces.

The excised left lower lobe (fig. 2) contained a large cavity at the base. This was composed of two compartments, the larger of which measured  $5 \times 4 \times 3$  cm. It communicated with many branches of the middle and posterior basal bronchi and was bridged by trabeculae of resistant lung. It contained air and muco-pus, and had a lining of ciliated columnar epithelium superimposed on scar tissue (figs. 3 and 4).

**Case 2.** A mentally backward orphan of 16. Pulmonary symptoms, which were slight, had no definite beginning, and probably dated from early childhood. He was brought up in a "home," was 18 months in hospital for tuberculosis, and spent  $2\frac{1}{2}$  years in a sanatorium, from which he ran away. Radiography revealed an extensive cystic condition in the right lung, and the heart drawn over to that side. There was no evidence of tuberculosis, and he was successfully treated by pneumonectomy.

The excised shrunken adherent right lung contained numerous cavities filled with air and muco-pus (fig. 5). The largest cavity occupied the greater part of the middle lobe and extended into the antero-lateral part of the upper lobe, where there were other cavities. A large cavity in the lower lobe communicated by small ostia with the three basal bronchi, and (more freely) with the dorsal bronchus. Both cavities were lined by ciliated epithelium (fig. 6). The bronchi communicating with the upper cavity were ectatic, but the basal bronchi communicating with the lower cavity were not. In some places there was "muscle fibrosis".

**Case 3.** A soldier of 24 whose history began at the age of 10 with pneumonia. An empyema developed, and was drained. Subsequently he remained well till he was 21 when he was in bed for several weeks with bronchitis. Four months before operation, when on active service, he had a febrile illness and was found to have a large cavity in the left lower lobe. This was followed by yet another attack. At Harefield Hospital the heart was found displaced to the same side as the cavity, and there was bronchiectasis, not only of the bronchi which opened into the cavity, but in the middle lobe on the opposite side. His general condition was good and there was no dyspnoea, but he had slight clubbing of the fingers and he brought up about 2 oz. of sputum a day. The affected lower lobe was excised and, although the bronchiectasis on the right side remained, his symptoms improved.

In this shrunken, adherent left lower lobe, a large cavity lined with ciliated columnar epithelium had formed an irregular ring round the periphery of the whole lobe (figs. 7 and 8). In the fibrosed lung surrounding the cavity there was an unusual amount of plain muscle. The bronchi entering the cavity were ectatic.

**Case 4.** A man of 53 with a "weak" chest since an attack of malaria 27 years before. He produced about a cupful of sputum a day, which was sometimes tinged with blood. He had had pneumonia three times, and a lung abscess had been drained. He was pale, thin and scoliotic. His blood pressure was low (85/60) and the blood sedimentation rate high (114). He died suddenly.

The cadaver exhibited several bed sores and there was a left 9th rib-resection scar. There was dorsal scoliosis to the left, on which side the lung was densely adherent. The left upper lobe contained a large healed cavity with a lining of ciliated columnar epithelium. This lay along the anterior margin and com-

municated with bronchi of the apical and anterolateral sectors. The other lung, which was adherent, emphysematous and oedematous, contained one large and several small emboli. There were two old infarcts in the spleen. A chronic peptic ulcer in the duodenum had ulcerated into the bile duct. The kidneys were fatty and the glomeruli infiltrated with amyloid.

**Case 5.** A married woman of 26, complaining of cough with 3 oz. of sputum daily. There was slight digital clubbing. Two years previously she had had right basal pleurisy and fluid had been withdrawn. A year later she had an attack which was thought to be due to a gall-bladder lesion. Four months later a right empyema was drained. At Harefield Hospital she had two further operations for empyema, one in the posterior part of the right base shortly after admission, the other on the same side anteriorly five months later. When she had been in hospital 7 months it was noticed in an X-ray film that the eye end of a darning needle was embedded in the hollow of the sacrum. It was rusty and in several pieces. Several unsuccessful attempts were made to remove it, and the last of these was followed by cellulitis and death.

At post-mortem a large abscess cavity was found in the pelvis which communicated with the rectum. This was associated with pulmonary and portal pyæmia. The sacrum showed suppurative osteomyelitis, and the needle was found embedded in a granulation tissue in its anterior surface. The gall bladder contained some pigment calculi. The epithelialised cavity, which had multiple bronchial communications, was situated in the posterior part of the adherent right base. It was not completely epithelialised, and was being drained by a rubber tube.

### *Summary*

Cavities in the lung with a respiratory epithelial lining are not necessarily congenital: they are sometimes epithelialised abscess cavities. In two cases (already reported) typical lesions were observed by repeated bronchography to arise in parts of the lung which were previously normal. In them, and in most of the present cases, the history began with symptoms suggestive of pneumonia or lung abscess.

The two features which most clearly distinguish healed cavities are:—(1) communication with multiple bronchi (indicative of ulceration) and (2) trabeculae of lung which have resisted the destructive process.

The ciliated epithelium lining healed cavities is usually superimposed on scar tissue. Persistent cavities become lined with epithelium of ciliated type when conditions for healing are favourable. When healing is delayed or repeatedly interrupted the epithelium is squamous.

The lesions in certain cases of "bronchiectasis" are healed abscess cavities.

I wish to thank Mr Holmes Sellors and Mr Vernon Thompson for the surgical specimens. To them and also to Dr L. G. Blair and Dr D. Houghton I am indebted for much stimulating discussion. The recognition of the condition was a co-operative effort, and in the first instance was largely dependent on radiography. I am also grateful to Professor W. D. Newcomb for helpful criticism. The photographs and sections were made by Mr W. Pereira.

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# THE INFLUENCE OF CULTURAL CONDITIONS ON THE MORPHOLOGY OF *BACTERIUM AERO-* *GENES* WITH REFERENCE TO NUCLEAR BODIES AND CAPSULE SIZE

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(PLATES XXXVII-XXXIX)

## LITERATURE

THE uniform staining usual in bacteria is often interpreted as indicating that the nuclear substance is evenly distributed throughout the cell; Imsenecki (1940) regards a "diffuse nucleus" as a primitive stage in nuclear evolution. On the other hand, the presence in bacteria of a discrete nuclear structure has been considered necessary to explain the constancy of transmission of hereditary characters (Lindegren, 1935) and the results of experiments on the lethal effect of ionising radiations (Holweck, 1929; Lea, 1946). Moreover, microscopic demonstration of nucleus-like intracellular bodies has been claimed. In a few cases these have been shown by ordinary staining methods (Stoughton, 1929; Allen, Appleby and Wolf, 1939; Peshkov, 1944-45), and more regularly by the Feulgen method (da Cunha and Muniz, 1929; Kužela, 1932; Stille, 1937; Piekarski, 1937, 1940; Neumann, 1941; Knaysi, 1942), by staining after hydrolysis with hydrochloric acid (Robinow, 1941-42) and by staining after treatment with the enzyme ribonuclease (Tulasne and Vendrely, 1947). Reasons for believing that these bodies are nuclei or chromosomes include their great affinity for basic dyes, their positive reaction to the Feulgen test for desoxyribonucleic acid, their selective absorption of ultraviolet radiation about 0.27 microns in wavelength (Piekarski, 1938), their loss of dye-affinity on treatment with desoxyribonuclease (Tulasne and Vendrely), their distinction from volutin by their resistance to hydrolysis with hydrochloric acid and persistence during starvation (Knaysi), and, most convincing of all, their occurrence in all cells at all stages of growth, their appearance of undergoing division in relation to cell division, their central position in the cell and their relation to the cytoplasmic boundaries and transverse septa (Robinow, 1941-42, 1943-44, 1945). Studies with the electron microscope have for the most part failed to reveal intracellular structures. However, by culture in a nitrogen-free medium (a solution of glucose and sodium acetate), Knaysi and Baker (1947) were able to render *Bacillus mycoides* more transparent to the electron beam so that relatively opaque intracellular bodies could be distinguished.

The influence of cultural conditions on capsule formation has not been adequately studied. The importance of serum and other native animal proteins has been frequently emphasised, indeed perhaps over-estimated. The dependence of capsule formation on the presence of carbohydrate in the medium has been shown for pneumococci and streptococci by Buerger (1907) and Morison (1940),

for *Bacterium coli* and salmonella organisms by Morgan and Beekwith (1939), and for *Bacterium friedländeri* by Hoogerheide (1939), who found that the addition of sugar to peptone media resulted in larger capsules, whereas small amounts of sugar were exhausted and capsules then diminished. In a sugar-containing synthetic medium, capsules were largest when growth was limited by a deficiency of phosphate.

### METHODS

The observations reported in this paper were made with 24 strains of *Bact. aerogenes* recently isolated from faeces, water, milk and air. The basis of the experimental media was 1 per cent. well-washed agar, 0.2 per cent. NaCl, 0.1 per cent.  $K_2SO_4$  and 0.025 per cent.  $MgSO_4$  in distilled water (pH about 6.0). The nutrient substances added to this base were either carbohydrate and peptone (Evans's bacteriological peptone solution at pH 7.4), or carbohydrate, ammonium sulphate and phosphate (a mixture of 1 part  $KH_2PO_4$  and 3 parts  $Na_2HPO_4$ , giving pH 7.3). In most tests the carbohydrate was sucrose; in some it was citric acid neutralised to pH 6.0 by addition of sodium hydroxide. When buffering without use of a high concentration (e.g. 1 per cent.) of phosphate was desired, either 1 per cent. calcium carbonate powder or 0.3 per cent. sodium bicarbonate was added to the medium and the plate incubated in a large sealed jar containing air plus 20 per cent. carbon dioxide; this  $NaHCO_3$ - $CO_2$  system was calculated to buffer at about pH 7.4. All chemical substances used were of analytical reagent quality. Glassware was thoroughly rinsed with distilled water on each occasion before use. Agar plates were inoculated to give confluent growth with a loopful of a suspension from a 21-hour nutrient agar culture. The plates were incubated aerobically at 37° C. for 24 hours. The pH of cultures was observed at room temperature by direct application of a glass electrode and by adding appropriate indicator dyes.

For demonstration of nuclear bodies, fairly thick smears were fixed by heat, stained for 1-2 minutes with 1 per cent. aqueous methyl violet, rinsed, blotted, dried and mounted in oil; these smears were not hydrolysed with hydrochloric acid. For "vital staining" in wet films, a drop of bacterial suspension was placed on a slide and firmly pressed under a coverglass which bore near one edge only the dried deposit of a smear of alcoholic methyl violet; part of the film was examined near to, but not directly under, the dye-bearing part of the coverglass. Volutin granules were observed in smears stained by Albert's method as modified by Laybourn (1924). Capsules were observed in very thin wet India ink films (Rowland, 1914); their transverse diameters, including the breadth of the bacillus together with the capsular layer on either side of it, were measured with an eyepiece micrometer. Measurements were made in parts of the film where the organisms were just gripped between slide and coverglass, not in the thicker parts where they drifted about freely and were partly overlaid by the ink, nor in the thinnest parts where capsule size appeared to be exaggerated by flattening. Dry-film methods of demonstration were not considered suitable for measurement of capsules on account of the variable and indeterminate shrinkage which may occur.

### EXPERIMENTAL OBSERVATIONS

#### *Dependence of morphology on composition of medium*

In heat-fixed methyl violet-stained smears, not hydrolysed with acid, three morphological types were distinguished: "normal," "nuclear" and "abnormal". On media with relatively little carbohydrate the growths were creamy and opaque and the cells were "normal": that is, they showed uniform staining without evidence

of nuclear bodies and possessed small capsules. On media with an excess of carbohydrate the growths were mucoid and transparent and the cells were "nuclear": that is, they showed darkly stained nuclear bodies within pale cytoplasm and usually possessed large capsules. The term "intermediate" was applied to growths containing a mixture of "normal," "nuclear" and transitional cells. On certain media growth was inhibited and consisted of giant pleomorphic cells designated "abnormal". Tables I-III summarise the observations made. Each observation was confirmed in at least three separate experiments, in each of which at least four strains were cultured. In many of the experiments all 24 strains were cultured.

TABLE I

*Morphology of 24-hour growths on media containing carbohydrate and peptone without additional buffering agent*

Medium				24-hour growth			
No.	Sucrose (per cent.)	Peptone (per cent.)	pH	Amount	pH	Cell morphology	Capsule diameter (microns)
1	0	1	7.5	+++	8.5	normal	1.1½
2	0	0.1	6.5	++	7.5	"	1.1½
3	0	0.01	6.0	+	6.5	"	1.1½
4	0	0.001	6.0	±	6.0	"	1.1½
5	0.01	1	7.5	+++	8.5	normal	1.1½
6	0.01	0.1	6.5	++	7.5	"	1.1½
7	0.01	0.01	6.0	++	5.5	intermediate	2.4
8	0.01	0.001	6.0	±+	5.0	nuclear	3.6
9	0.1	1	7.5	++++	8.5	normal	1.2
10	0.1	0.1	6.5	++++	6.0	intermediate	2.4
11	0.1	0.01	6.0	++++	4.5	nuclear	3.6
12	1	1	7.5	+++++	7.0	intermediate	1½-4
13	1	0.1	6.5	+++++	4.5	nuclear	3.6
14	1	0.01	6.0	+++	4.5	"	3.6
	Citric acid (per cent.)						
15	1	1	6.5	+++	9.0	normal	1.1½
16	1	0.1	6.0	++	8.5	"	1.1½
17	1	0.01	6.0	++	7.5	nuclear	2.4
18	1	0.001	6.0	+	6.5	"	2.4

±, +, ++ etc. denote increasing amounts of growth.

The terms used to describe cell morphology have the following meanings:—

"normal" = uniform staining, no visible nuclei, small capsules.

"nuclear" = dark nuclei in pale cytoplasm; capsules usually large.

"abnormal" = giant and pleomorphic.

"intermediate" = mixture of normal, nuclear and transitional.

*Carbohydrate-peptone media.* Morphology on media containing carbohydrate and peptone was "normal" or "nuclear", depending on whether the carbohydrate-peptone ratio was low or high (table I).



## PLATE XXXVII

FIG. 1.—Strain 4; 2 hours on nutrient agar; smear hydrolyzed with acid and stained with Giemsa's solution (Robinow's method). Normal bacilli and one filamentous cell show dark-stained nuclear bodies in weakly stained cytoplasm.

FIG. 2.—Strain 4; 24 hours on nutrient agar; smear stained as in fig. 1. This shows dark-stained nuclear bodies, but less clearly than in the larger cells of the 2-hour growth.

FIG. 3.—Strain 3; 24 hours on medium 9; heat-fixed, methyl violet-stained smear. "Normal" bacilli uniformly stained.

FIG. 4.—Strain 4; 24 hours on medium 13; heat-fixed, methyl violet-stained smear. "Nuclear" bacilli, showing dark-stained nuclear bodies in pale cytoplasm outlined by stained extracellular material.

FIG. 5.—Strain 3; 24 hours on medium 22; heat-fixed, methyl violet-stained smear. "Nuclear" bacilli, showing dark-stained nuclear bodies and pale cytoplasm but no stained extracellular material.

FIG. 6.—Strain 4; 24 hours on medium 14\*; heat-fixed, methyl violet-stained smear. "Nuclear" bacilli, showing dark-stained nuclear bodies and pale cytoplasm but no stained extracellular material.

FIGS. 7 and 8.—Strain 2; 24 hours on medium 12\* + 1 per cent.  $\text{CaCO}_3$ ; heat-fixed, methyl violet-stained smear. Mostly "nuclear" bacilli, showing dark nuclear bodies, pale cytoplasm and stained extracellular material outlining cells and forming a median transverse septum in some; each figure shows two transitional cells with faint outlining and dark-stained volutin granules but not nuclear bodies.

All figures  $\times 3750$

\* Media marked with an asterisk contained lactose instead of sucrose.

PHOTOMICROGRAPHS OF *BACTERIUM AEROGENES*



FIG. 1.



FIG. 2.

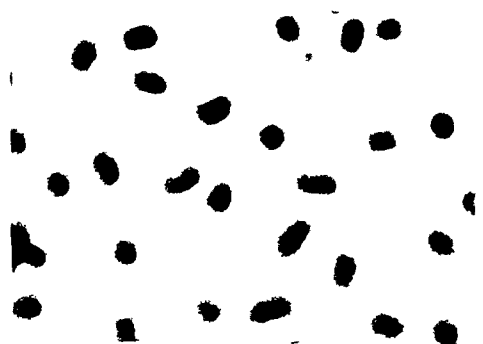


FIG. 3.

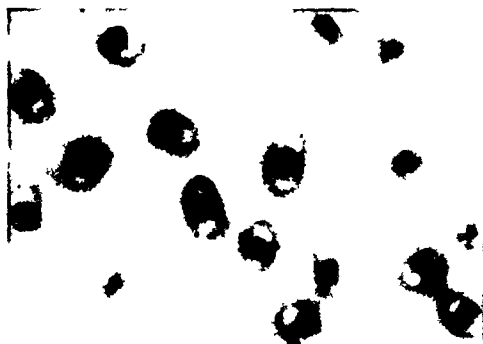


FIG. 4.



FIG. 5.

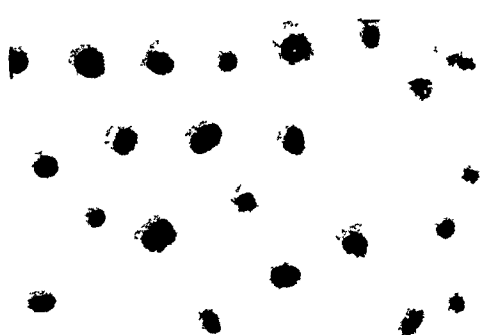


FIG. 6.



FIG. 7.

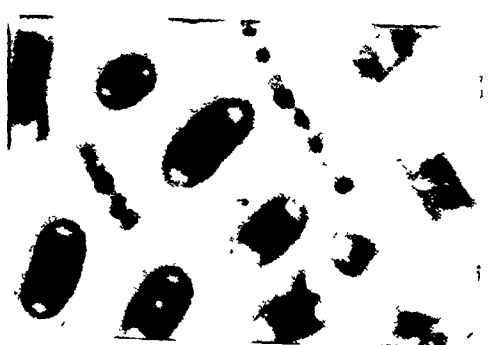


FIG. 8.



with 1 per cent.  $\text{CaCO}_3$  resulted in growth failure and the formation of "abnormal" spherical and oval cells which were often very large; growth failure could be avoided if this  $\text{CaCO}_3$ -buffered peptone-deficient medium was either incubated with 20 per cent. carbon dioxide or supplemented with phosphate.

*Synthetic media.* Peptone could be replaced by a combination of phosphate and ammonium sulphate in adequate amounts. In such synthetic media, "normal" growth was obtained when both the carbohydrate-phosphate and the carbohydrate-ammonium salt ratios were low, and "nuclear" growth when either of these ratios was high (tables II and III). In the presence of abundant ammonium

TABLE III

*Morphology of 24-hour growths on media containing carbohydrate, phosphate and ammonium sulphate without additional buffering agent*

Medium					24-hour growth			
No.	Sucrose (per cent.)	Phosphate (per cent.)	Ammonium sulphate (per cent.)	pH	Amount	pH	Cell morphology	Capsule diameter (microns)
37	0.1	1	1	7.0	+++	7.0	normal	1.2
38	0.1	1	0.1	7.0	+++	7.0	"	1.3
39	0.1	1	0.01	7.0	+++	7.0	intermediate	1½-5
40	0.1	1	0.001	7.0	++	7.0	nuclear	3.6
41	0.1	1	0.0001	7.0	+	7.0	"	3.6
42	0.1	0.01	1	6.5	+	4.0	abnormal	small
43	0.1	0.01	0.1	6.5	+	4.0	"	"
44	0.1	0.01	0.01	6.5	+	4.0	"	"
45	0.1	0.01	0.001	6.5	±++	4.5	nuclear	3.7
46	0.1	0.01	0.0001	6.5	±+	5.5	"	3.7
47	0.1	0.1	0.1	7.0	+++	6.0	normal	1.3
48	0.1	0.01	0.1	6.5	+	4.0	abnormal	small
49	0.1	0.001	0.1	6.0	+	4.0	"	"
50	0.1	0.0001	0.1	6.0	±+	4.5	nuclear	1.4
	0.1	0.00001	0.1	6.0	+	4.5	"	2.5
Citric acid								
51	1	0.01	0.1	6.5	++	8.5	normal	1-1½
52	1	0.001	0.1	6.0	++	8.0	"	1-2½
53	1	0.0001	0.1	6.0	++	7.0	nuclear	1½-5
54	1	0.00001	0.1	6.0	+	6.5	"	1½-5
55	1	0.01	0.1	6.5	++	8.5	normal	1-1½
56	1	0.01	0.01	6.5	++	8.0	"	1-1½
57	1	0.01	0.001	6.5	++	7.0	nuclear	1½-4
		0.0001	0.0001	6.5	+	6.5	"	1½-4

sulphate, morphology was "normal" with sucrose-phosphate ratios of 1 to 10, 1 to 1 and 10 to 1, "intermediate" with a ratio of 100 to 1, and "nuclear" with ratios of 1000 to 1, 10,000 to 1 and 100,000 to 1. In the presence of abundant phosphate, morphology was "normal" with sucrose-ammonium sulphate ratios of 1 to 10 and 1 to 1, "inter-

mediate" with a ratio of 10 to 1, and "nuclear" with ratios of 100 to 1, 1000 to 1 and 10,000 to 1. Synthetic media containing citric acid gave similar results, 1 per cent. citric acid being equivalent to 0.01-0.1 per cent. sucrose (table III: media 51-57).

The reaction of the sucrose-containing synthetic media could be maintained at about neutrality by the addition of the  $\text{NaHCO}_3\text{-CO}_2$  system, of 1 per cent. phosphate or of 1 per cent.  $\text{CaCO}_3$ . The  $\text{NaHCO}_3\text{-CO}_2$  system was the best and most generally applicable buffer, allowing good growth on media of every composition. One per cent. phosphate was a satisfactory buffer in tests on variation in ammonium salt content of medium. Calcium carbonate proved a satisfactory buffer for media containing more than the smallest amount of phosphate. However, the addition of 1 per cent.  $\text{CaCO}_3$  to media containing only 0.00001 or 0.0001 per cent. added phosphate resulted in growth failure and formation of "abnormal" cells, giant spheres and ovoids (fig. 19). Similar inhibition of growth in phosphate-deficient media was caused by the addition of small amounts (e.g. 0.01 per cent.) of sodium hydroxide or sodium bicarbonate, giving a pH of 7.0-8.0. Such inhibition of growth could be prevented by incubation in 20 per cent. carbon dioxide.

Sucrose-containing synthetic media without added buffer gave growths similar to those on the corresponding buffered media only when their phosphate content was sufficient (0.1-1 per cent., as in media 38 and 47), or when growth and acid production were greatly limited by deficiency of phosphate or ammonium sulphate (media 45, 46, 49 and 50). Apart from these cases, acidity became so great in the unbuffered synthetic media (media 42, 43, 44 and 48; final pH about 4.0) that only scanty "abnormal" growths were obtained; these consisted mainly of long bacilli with numerous volutin granules and a few filaments showing branching, spindle-shaped swellings or protoplasmic protrusions (fig. 21); some of the latter forms were identical with the zygospor-like "megalomorphs" of Wahlin and Almaden (1939). Numerous megalomorphs were produced by all 24 *Bact. aerogenes* strains when cultured for 2-5 days in the liquid media of Wahlin and Almaden (figs. 20 and 22). When these liquid media were buffered with 1 per cent.  $\text{CaCO}_3$  or 1 per cent. phosphate, growth was heavier and megalomorphs were not formed, which leads to the conclusion that megalomorphs are involution forms produced as a result of an unfavourably acid reaction.

#### *Cytological observations*

"Nuclear" bacilli. In heat-fixed, methyl violet-stained smears "nuclear" bacilli showed one or two centrally placed, dark-blue stained nuclear bodies within very weakly stained cytoplasm; the cytoplasm was usually surrounded and outlined by red-violet stained extracellular material, probably capsular (figs. 4-8).



## PLATE XXXVIII

- FIG. 9.—Strain 3; 24 hours on medium 9; unfixed wet film with India ink. "Normal" bacilli with small capsules.
- FIG. 10.—Strain 3; 24 hours on medium 13\*+1 per cent.  $\text{CaCO}_3$ ; unfixed wet film with India ink. "Nuclear" bacilli with large capsules.
- FIG. 11.—Strain 3; 24 hours on medium 13; unfixed wet film "vitally" stained with methyl violet. "Nuclear" bacilli completely masked by heavily stained extracellular material.
- FIG. 12.—Same film as in fig. 11, with subsequent addition of India ink. Extracellular material now decolourised; capsules outlined by ink are bigger than stained extracellular zones in fig. 11.
- FIG. 13.—Strain 23; 24 hours on medium 45+1 per cent.  $\text{CaCO}_3$ ; unfixed wet film "vitally" stained with methyl violet. "Nuclear" bacilli exposed for a few minutes to high dye concentration are masked by heavily stained extracellular material.
- FIG. 14.—Strain 19; 24 hours on medium 45+1 per cent.  $\text{CaCO}_3$ ; unfixed wet film "vitally" stained with methyl violet. "Nuclear" bacilli exposed to lower dye concentration than those in fig. 13, showing uniformly stained protoplasm and weakly stained extracellular structure.
- FIGS. 15 and 16.—Strain 3; 24 hours on medium 14; unfixed wet film "vitally" stained with methyl violet. "Nuclear" bacilli exposed for 3 hours to low dye concentration, showing dark-stained nuclear bodies in paler cytoplasm; extracellular material is seen weakly stained in fig. 15, but not stained in fig. 16.

All figures  $\times 3750$

\* Media marked with an asterisk contained lactose instead of sucrose.

PHOTOMICROGRAPHS OF *BACTERIUM AEROGENES*



FIG. 9.



FIG. 10.

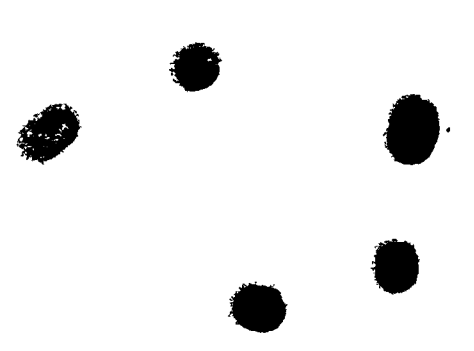


FIG. 11.



FIG. 12.

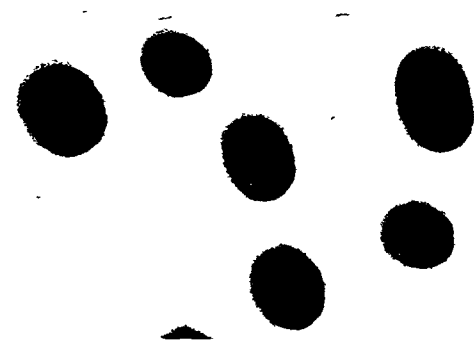


FIG. 13.



FIG. 14.

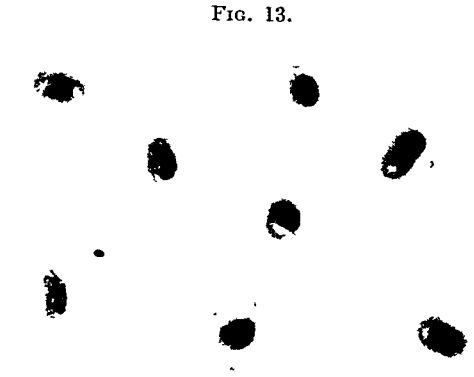


FIG. 15.

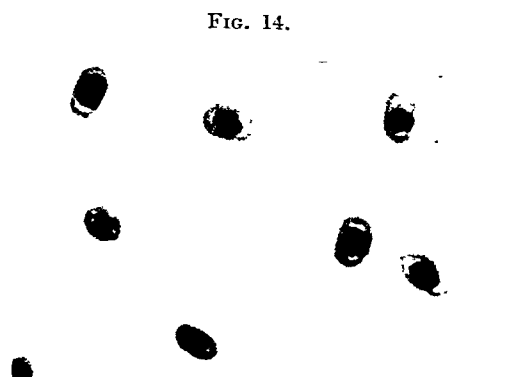


FIG. 16.





Because the "nuclear bodies" seen in dried and heat-fixed smears might be artefacts due to shrinkage, much importance was attached to their demonstration in unfixed wet films "vitaly-stained" with methyl violet. This was achieved for cultures on various media (media 13, 14, 17, 22 and 40), but not readily and not regularly. When the cells were first exposed to the dye a very heavy staining of the extracellular material completely masked the internal structures (figs. 11 and 13). However, after some minutes or hours had elapsed a few cells were usually found, distant from the dye-bearing part of the coverglass, which possessed unstained or weakly stained extracellular material and showed dark-stained nuclear bodies in weakly stained cytoplasm (figs. 15 and 16); in addition, there were many unmasked bacilli which showed uniformly stained protoplasm (fig. 14).

The "vital"-staining method showed clearly the location of the stainable extracellular material. While still unstained the bacilli showed a refractile outline, presumably the "cytoplasmic membrane" of Knaysi (1946); the capsules were invisible. As the methyl violet reached the cells it was seen to cause rapid and heavy staining of a hitherto invisible structure which was external to the cytoplasmic membrane. The stained extracellular structure had a smooth or spiky outline, and a transverse diameter usually from  $1\frac{1}{2}$  to 4 microns. Although presumably capsular, it did not correspond to the whole capsule. If India ink was added to the edge of a methyl violet-stained wet film, it could be observed diffusing in, decolourising the stained structures and outlining the capsules; the capsules were clearly bigger than the stainable extracellular structures of the same cells, for instance 4-5 microns broad as compared with  $1\frac{1}{2}$  microns (cf. fig. 11 with fig. 12).

Unfixed wet films with India ink showed that unusually large capsules were formed by all twenty-four strains of *Bact. aerogenes* on all the media shown in the tables as giving "nuclear" growth. Capsules were largest on the peptone media with high sucrose content and on ammonium salt-deficient sucrose synthetic media. Different cells ranged in breadth from 3 to 7 microns, but were mostly from 4 to 5 microns broad. Capsules were smaller, 2-5 microns, on the phosphate-deficient sucrose synthetic media, especially when unbuffered, and on the citric acid media. There was surprisingly little variation in capsule size between different strains; two strains regularly formed smaller capsules than the others. Where the India ink happened to flow past stationary bacilli, a gradual disintegration of the capsules could be seen; strands of capsular material were drawn out, became detached and drifted away. The outlining by ink particles of even the finest of these strands shows that the ink particles must closely abut on the capsular substance and are not repelled to a distance by the operation of physical forces as suggested by Sokhey (1940). In Albert-stained smears the nuclear bodies took up the

green dye and only rarely stained metachromatically; they thus appeared not to be volutin granules.

The "nuclear" cells were not involution forms; they were fully viable. "Nuclear" bacilli from a 24-hour culture on 1 per cent. sugar-0.1 per cent. peptone medium were inoculated on to a nutrient agar block and observed with an incubated microscope. Almost all these bacilli showed rapid growth; increase in cell size was apparent and the first division was often completed within two hours. The morphology of subcultures of "nuclear" bacilli on nutrient (meat extract and peptone) agar was observed. Methyl violet-stained smears showed that within 2 or 3 hours of subculture the cytoplasm became strongly stained so that the nuclear bodies were masked and "normal" morphology assumed. Albert-stained smears showed that in certain cases deposition of volutin played a part in this morphological transition. When "nuclear" bacilli from a phosphate-deficient synthetic medium (media 22, 49 and 53) were subcultured on nutrient agar, metachromatic material was rapidly deposited about the nuclear bodies. Within half an hour most bacilli showed 1-4 metachromatic granules. After about 3 hours these disappeared. In contrast, "nuclear" cells from synthetic media containing little ammonium sulphate but abundant phosphate (media 26, 40, 45 and 56) did not deposit volutin when subcultured.

"Normal" bacilli. In heat-fixed, methyl violet-stained smears "normal" bacilli showed intense uniform staining (fig. 3), or bipolar and peripheral staining; nuclear bodies could not be distinguished within them; the capsular material was unstained and invisible. When "normal" bacilli were stained after hydrolysis (Robinow's method) they showed differentiation of nuclear bodies (figs. 1 and 2). In unfixed wet films "vitaly" stained with methyl violet, "normal" bacilli stained at first only lightly and only at the periphery (fig. 17). The peripheral staining layer appeared to be just external to the cytoplasmic membrane and equivalent to the much broader capsular staining zone observed in "nuclear" bacilli. On longer exposure to the dye the bacilli came to stain darkly and uniformly; only rarely was there apparent differentiation of nuclear bodies (fig. 18). Unfixed wet films with India ink showed that the capsules of "normal" bacilli were much smaller, mainly about  $1\frac{1}{2}$  microns in diameter, than those of "nuclear" bacilli (cf. figs. 9 and 10). In Albert-stained smears "normal" bacilli did not show volutin granules.

## DISCUSSION

The intracellular bodies demonstrated in ordinary stained smears of *Bact. aerogenes* grown on media of high carbohydrate content seem to be identical with the nuclear bodies described by Robinow (1941-42) and others as being present in many common bacteria. The nuclear nature of the bodies now observed is borne out by their high



## PLATE XXXIX

FIGS. 17 and 18.—Strain 3; 24 hours on medium 1; unfixed wet films "vitaly" stained with methyl violet; "normal" bacilli. Fig. 17 shows peripheral staining given after a few minutes' exposure to the dye. Fig. 18 shows intense uniform staining given after longer exposure (1 hour); also in two cells at extreme left and in one at right side apparent differentiation of nuclear bodies.

FIG. 19.—Strain 21; 24 hours on medium 49+1 per cent.  $\text{CaCO}_3$ ; unfixed wet film "vitaly" stained with methyl violet; "abnormal" spherical and irregularly shaped giant cells.

FIG. 20.—Strain 4; 4 days in the 1 per cent. glycerol liquid synthetic medium of Wahlin and Almaden; smears hydrolysed with acid and stained with Giemsa's solution (Robinow's method); "abnormal" filamentous cells, including one with a spindle-shaped swelling containing tangled strands of nuclear material.

FIG. 21.—Strain 4; 24 hours on medium 43; unfixed wet film "vitaly" stained with methyl violet; "abnormal" cells, filamentous and branching.

FIG. 22.—Strain 4; 4 days in the 1 per cent. glycerol liquid synthetic medium of Wahlin and Almaden, supplemented with 0.1 per cent. peptone and 0.1 per cent. meat extract; heat-fixed, methyl violet-stained smear; "abnormal" filamentous cells containing volutin granules; one filament bears a spindle-shaped swelling.

All figures  $\times 3750$

\* Media marked with an asterisk contained lactose instead of sucrose.

PHOTOMICROGRAPHS OF *BACTERIUM AEROGENES*

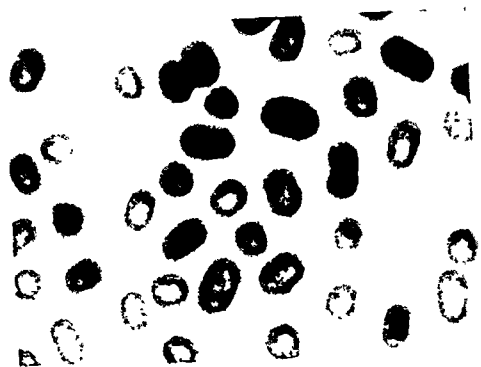


FIG. 17.



FIG. 18.



FIG. 19.



FIG. 20.



FIG. 21.



FIG. 22.



affinity for basic dyes, their position in the cell and their constant presence in all cells of a culture; they are not volutin granules since they do not give the metachromatic staining reaction; they are not shrinkage artefacts since they may be seen in unfixed wet films "vitaly" stained; they do not represent the shrunken protoplasm of degenerate or plasmolysed cells since on subculture the cells reproduce rapidly and normally. Knaysi and Baker explained the unmasking of nuclear bodies in *Bacillus mycoides* grown in a nitrogen-free medium as being due to exhaustion of the intensely staining cytoplasmic ribonucleic acid. A similar explanation possibly applies in the present studies of *Bact. aerogenes*, a deficiency of either phosphate or nitrogenous nutrient preventing accumulation of ribonucleic acid in the cytoplasm.

The observations of Hoogerheide that capsule formation by *Bact. friedländeri* depends upon the presence of carbohydrate and that capsules are larger when growth is limited by deficiency of phosphate are paralleled by the present findings. Moreover, I found that a deficiency of nitrogenous nutrient has the same effect as a deficiency of phosphate in causing the formation of large capsules and I suggest that any nutritional deficiency capable of arresting growth before the available carbohydrate is exhausted in meeting energy requirements will, by conserving carbohydrate for synthesis of polysaccharide, lead to the formation of large capsules.

#### SUMMARY

The cell morphology of *Bact. aerogenes* was profoundly influenced by the nutrient balance and pH of the culture medium. Cells of an unusual type were formed in growths on media containing little peptone and much carbohydrate and in growths on synthetic media which were deficient in either phosphate or ammonium salt in relation to their carbohydrate content. These cells possessed exceptionally large capsules and in smears stained by ordinary methods showed deeply stained nuclear bodies within weakly stained cytoplasm. When subcultured on nutrient agar the "nuclear" cells grew and multiplied rapidly, becoming transformed within a few hours into uniformly staining cells of the "normal" type. Growth was inhibited and pleomorphic giant cells were formed under two different conditions, namely when a highly acid reaction developed and when a phosphate-deficient medium was rendered slightly alkaline.

I wish to thank Professor T. J. Mackie for the interest he has shown in this investigation, and Dr H. K. King for his advice on chemical problems.

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## FUNCTION AND ARRANGEMENT OF BACTERIAL FLAGELLA

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(PLATES XL-XLVII)

IN 1838 Ehrenberg observed that some large bacteria, for example *Monas (Chromatium) okenii*, moved by means of flagella. The same was observed by Cohn (1872) for *Spirillum volutans*, by Koch (1877) for *Spirillum undula* and by Migula (1897) also for *Spirillum volutans*. The flagella of living bacteria, however, were seldom visible until Zettnow, Loeffler and others successfully stained them, when it became evident that only motile bacteria possessed flagella and that the arrangement of flagella could be polar or peritrichous. Later, dark-field illumination made it possible to study the flagella of some living bacteria, as was first done by Reichert (1909) and later by Neumann (1925, 1928) and Neumüller (1927).

Flagella had thus come to be generally accepted as organs of bacterial motility when Pijper (1946, 1947) surprisingly stated his belief (reviving the old theory of van Tieghem, 1879) that flagella were not organs of locomotion, but only artefacts—useless appendages, polysaccharide twirls—the result, not the cause, of bacterial motility. My intention is to argue that the facts are against Pijper's view and I propose to challenge also his assertion (Pijper, 1930, 1931-32) that peritrichous flagellation does not exist, but that flagella are artefacts of the staining methods employed, and not, therefore, of any use for purposes of taxonomy. Pietschmann (1941-42) and others have also doubted the value of flagellar stains for the systematic classification of bacteria.

### TECHNICAL METHODS

The light source for this investigation was a Philips water-cooled, super-high-tension mercury lamp, S.P. 500 W, which is ideal for this type of work, because it does not need attention like a carbon arc lamp and gives a stronger light without heat rays. The intensity used was probably not much lower than in Pijper's work. With a magnification of 110 (objective used without ocular) photographs could be taken with an exposure of 1/5 second. Fig. 1, for example, was so taken and afterwards enlarged 9 times. 1/25 second was a little too short an exposure. The optics used were a Zeiss cardioid condenser

and Zeiss apochromat  $\times 60$ . For direct observation a Zeiss binocular Bitukni was used, with  $\times 15$  oculars. The camera was a Zeiss "Miflex"  $6 \times 9$  cm. Stained preparations were photographed with a Zeiss fluorite A  $1.3 \times 100$  and compensating ocular  $\times 20$ , enlargement  $\times 1000$ , the light source being a Philips high-tension mercury lamp PII 300 and wavelength  $365 \mu$  (Boltjes, 1946).

To slow down the movement of the micro-organisms and make the flagella visible, the spirilla were suspended in a 10 per cent. neutralised solution of gum arabic or gelatin and the bacteria in 2.5 per cent. solutions of these substances. The solutions were freed from colloidal particles by filtration through Seitz discs.

Staining was done with the Peppler mordant and the Zettnow silver ethylamine solution. The mordant is prepared by dissolving 20 g. of tannin in 80 ml. of water, to which is added, with continuous stirring, 15 ml. of a 2.5 per cent. solution of chromic acid in water. Before use the mordant is kept for 7 days at  $20^\circ \text{C}$ . The Zettnow silver ethylamine solution is prepared by dissolving 0.15 g. of  $\text{AgSO}_4$  in 60 ml. of water and adding ethylamine, with continuous stirring, until the precipitate formed is just dissolved. Films of the bacterial suspension are made on freshly annealed coverslips, fixed by heat, flooded with the filtered mordant and gently heated. After 10-15 minutes the mordant is washed off and the preparation dried and flooded with the silver ethylamine. This is heated till steaming and after 1-2 minutes washed off and the preparation dried.

#### EXPERIMENTAL OBSERVATIONS

The organisms studied were *Spirillum serpens*, *Proteus mirabilis* and *Salmonella typhi*.


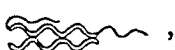
##### *Spirillum serpens*

I started with the spirillum because Pijper maintained that bacteria move, not by means of their flagella, but because their bodies make a gyratory undulatory movement or a spiral or screw-like motion. He was therefore of the opinion that bacteria should not be considered as rods but as spirals or, better, as helices. But one glance through the microscope reveals immediately, if one fixes attention on spirilla which have somehow stuck to the coverglass, that their flagella cannot be dead structures—mere artefacts or the result of the movement of the spirilla, for the flagella are turning, whipping and bending backwards and forwards at a speed and in a way that could never be caused by currents in the preparation. Migula (1897, p. 111) also had no doubt that *Spirillum volutans* is moved forward by its tail. In fig. 2 the moving flagella are seen as elongated loops. The observer gains the impression that the organism knows it has flagella and understands how to use them.

Looking at *Proteus* one does not get the same impression. Here the flagella always turn stubbornly to the right, and to change direction the whole apparatus is brought backward or forward. When the organism sticks, the flagella go on turning, but aimlessly and without co-ordination, perhaps because the organism usually consists of more than one individual, and they fail to co-ordinate their movements. If, however, they are swimming free in a very long train they work together in a way not unlike that of a rowing crew.


It is not easy to identify the type of movement shown by the flagella of spirilla. It seems to be a double movement—a spiral combined with a lashing movement. It is certain, however, that the flagella can turn to the left and to the right. When the spirillum swims, the forward flagellum is usually bent backwards but sometimes it is stretched forwards. The moving spirillum can reverse very quickly, and in this process the forward flagellum is stretched out straight, while the other is bent backwards.

The body of a stranded spirillum is clearly rigid and makes no motion at all, as it should do if the motility was produced in the way described by Pijper: only the flagella are working vigorously. When two organisms stick together, they remain in this position:—

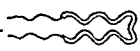
 , and never come into this position:—  ,

which would be expected if their bodies made spiral motions, since this would be the best way to get rid of each other. From this it will be clear that the body of the spirillum does not make any movement.

The following observations show that the flagella are really propelling the spirillum forward. Often the spirilla turn quickly

around an axis parallel to that of the microscope tube:—  .

This movement cannot be made without flagella. Once I saw a very long spirillum which was bent double. Yet it was quietly swimming

about thus:—  . This was one organism, for after some time it suddenly snapped straight. If the movement of the spirillum was caused by a spiral motion of the body, the bent spirillum could never move forward, because the motion of one half would oppose that of the other half. Also, I have often observed that clumps of spirilla could swim across the field of the microscope without a single body making a spiral movement. Such clumps can move only by the movement of the flagella.

Reichert (1909), who also studied the spirilla, said that the spirilla are left-handed and the flagella right-handed spirals. He and Bütschli (1883-87) were quite right in saying that if the flagella made a right-hand motion, a left-handed spiral body would support their movement. However, I could not confirm that the spirilla are left-handed spirals. I have already mentioned that the flagella can turn to the right and to the left, and only this can explain how a spirillum may move forward without turning about its axis; and also the fact that spirilla often change the direction of turning without reversing the direction in which they swim, a phenomenon observed by others in the laboratory besides myself. It was too difficult to make out if the spirilla were left- or right-handed spirals, but my opinion is that there were as many left- as right-handed spirilla.

*Proteus mirabilis*

The strong illumination of the dark field seemed to do very little harm to the spirilla. They could be observed for quite a long time before they became less motile. *Proteus* on the other hand was rather sensitive to strong light. When it entered the light spot of the dark field the motion was often retarded immediately, and in a gum arabic solution the bacteria soon came to rest, with only the flagella turning slowly round. Soon this motion also stopped. When a glass cell with a solution of aurantia (5-10 mg./100 ml. water) was placed before the light source the flagella began to revolve again within a short time, first slowly, but gradually gathering speed and the bacteria moved on again. If the filter was pulled away the motion stopped immediately, and after its replacement the motion started again. This could be repeated 5-10 times.

When the bacteria swam in gelatin, the harmful effect of the illumination was much smaller. This experiment clearly shows that the spiral movement of the flagella cannot be caused by currents, that the bacterium is propelled forward by the flagella and, therefore, that the flagella are really organs of locomotion.

Fig. 1 shows a train of *Proteus* cells in full movement in a 5 per cent. solution of gelatin. The flagella lie close to the body and are therefore difficult to see. Only at the rear end can the flagella be seen clearly. In most cases there are two, as is shown here, but there may be three. Looking at the posterior flagella one sees that the body of the bacterium is rotating slowly around its axis to the right or to the left. Often the rotation is suddenly reversed, and it also happens that the rotation is not completed, so that the bacterium swings around its axis from right to left. It is very clear that the body itself does not contribute to the movement.

I had just finished the first draft of this manuscript when my attention was drawn to an article by Ørskov (1947), in which he described a very elegant method of demonstrating the activity of *Proteus*. An ordinary nutrient agar plate with 1-2 per cent. agar is inoculated with *Proteus* and incubated for a few hours at 30° C. As soon as the bacteria begin to swarm, small pieces of agar with the bacteria in position are cut out and put on a slide. A coverglass on which a drop of filtered India ink is spread out with another coverglass is dropped on the piece of agar. The bacteria in some parts of the preparation can then be seen to trace a clearly visible path through the India ink. With an oil-immersion lens it can be seen that the small ink particles are swept to the rear and sometimes there is a faint glittering along the bacteria. Especially at the posterior end of the bacterium the ink particles are obviously in a strong current. The flagella themselves cannot be seen, but they are clearly the cause of this current. This is shown when the bacteria remain on the same spot; the particles are still in lively motion and after some time the

flagella have swept a clear field around the bacterium (fig. 33). The other lines on the picture are the paths drawn by the bacteria. I observed that if one makes the preparation in the way described one has to wait rather a long time before the bacteria stop, but if the agar is first flooded with a 0.2 per cent. solution of methylene blue the result shown in fig. 33 appears within a few minutes.

### *Salmonella typhi*

The flagella of *Salm. typhi* move in the same way as those of *Proteus*. The motion of these two bacteria is also very much alike, but *Salm. typhi* waggles a little more than *Proteus*. Curiously, typhoid bacilli seem to have some difficulty in passing each other and I could not make out the cause of this phenomenon, which was not observed with the spirilla or *Proteus*. I also observed that dying typhoid bacilli form clumps, which the other bacteria do not. It was clear that the flagella are organs of motion and that the body itself does not initiate movement.

### DISCUSSION OF PIJPER'S ARGUMENTS IN THE LIGHT OF THESE OBSERVATIONS

1. Pijper observes that none of the electron-microscope pictures show flagella as connected with the inner part of the cell or as traversing the cell wall. At the time he wrote, this was indeed true; but the fine photographs of van Iterson (figs. 40-42) show in such a convincing way\* the true nature of the flagella that this argument has lost its value.

It seems to me that Pijper overrates the value of dark-ground microscopy. By its contrast effect it may reveal small particles which are overlooked in the light field, but it should be kept in mind that the resolving power is not increased. Further, it is of importance to know, as Rayleigh computed, that the intensity of the scattered light is proportional to the quadrate of the volume. For a sphere, the intensity is proportional to the sixth power of the radius. It is also clear that the visibility rapidly diminishes with the size of the object. One would think, perhaps, that increasing the intensity of the light source would very much enlarge the power of the dark-field illumination, but this has also the effect of rendering the background less dark. In addition, large particles radiate much more strongly, so that contrast diminishes and small objects remain invisible.

2. Pijper (1946, p. 330) writes: "The only place left where flagellar appendages can originate is the slime layer" and: "This would explain why in stained preparations there is so frequently a gap between cell wall and flagella". Pijper illustrates his point of view with an acceptable sketch of a spirillum with long slimy capsule

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\* Conn and Elrod (1947) also find these electron photomicrographs very convincing.

material, but it is inconceivable that slimy material on a *Proteus* might eventually arrange itself along the full length of the body in such neat spirals as the flagella of this bacterium; therefore this explanation of the origin of the flagella cannot be accepted.

It is possible to give another explanation of the gap between the bacterium and the flagella. The gap arises if, during the drying process, the flagella stick to the coverglass so soon that they are torn off from the bacterium when it shrinks. Since the bacterium shrinks more in length than in breadth, it is natural that separation should occur more readily and is better seen with polar than with peritrichous flagella (fig. 5), which seldom separate. If a slime layer were the cause of this separation, the gap would be observed with the same frequency for peritrichous and polar flagella, and would be of about the same size in each case.

3. The flagella and the slime layer have similar staining reactions. This is certainly true, for it is obvious that flagellar stains are not in the least specific. This is the reason why slime material has sometimes been looked upon as flagella. Pijper (1946) even says that there is a significant confusion among mucous threads, slime and flagella, and concludes from this that all flagella are only slimy artefacts. But this conclusion is wrong, for it is sometimes possible to recognise pseudo-flagella (e.g. Thjotta and Käss, 1946) and there is sometimes a wide difference between them and true flagella. For instance, nobody would mistake the threads of capsule material sometimes formed by *Nitrosomonas* (Boltjes, 1935) for the flagella of this organism, while fig. 27, of *Hyphomicrobium vulgare* (Boltjes, 1936), shows clearly which are the flagella and which are not.

4. Pijper is of the opinion that there is only a superficial similarity between animal and bacterial flagella and that there is therefore no reason to accept bacterial flagella as organs of locomotion. In this connection it is worth noting an observation of Neumann (1925), which I can confirm, that *Proteus*, like *Paramoecium*, can shoot off its flagella while the body of the organism remains intact. This explosion is a very curious sight: the shot-off flagella, because of their spiral shape, are quickly revolving and look like minute spirilla, and they may even traverse nearly the entire field of the microscope. In my opinion this proves that the similarity is more than superficial, and it also shows that the flagella cannot be the fringe of slimy capsule material, since this could not be thrown off without disruption of the whole cell. And the cell does not explode.

5. Pijper says that his cinemicrographic pictures show clearly that the body makes a spiral motion. If this were true, it would be the crucial argument; but we shall see that this is not the case. According to Pijper both spirillum and bacterium move forward by a gyratory undulatory movement, so that they move in the same way as the flagella of *Proteus*. An acceptable explanation of the mechanism of flagellar action has already been given by Bütschli (1883-87) and

Reichert (1909). Bütschli supposes that a contraction passes along as well as around the flagellum. To observe the rotation of the body, a small particle—a bacterium for instance—must be attached to it. When this observation was made, it appeared that the body of the spirillum always turned in the same direction as the observed apparent spiral movement, which proves that the body of the spirillum is a rigid body turning around its axis and propelled by its flagella. Pijper's film (Pijper, 1946, fig. 20) also shows that the body of the bacterium itself does not move, for if the bacterium were making a spiral movement, the bend caused by the passing circulating wave would not remain in the same place on the bacterium, but would move along it. Pijper's picture shows clearly that the bend remains always in the same place and therefore it cannot prove that the bacteria are making a spiral motion. From this it is also clear that we must still consider the bacterial body as rigid, and conclude that it moves only by the action of its flagella.

6. Pijper says truly that flagella are not essential for motility, but it seems strange to compare the slow creeping motion of, for instance, blue algae, diatoms, *Beggiatoa alba* and Myxobacteriales with the rapid motion of the free-swimming bacteria. It seems unlikely that a mechanism similar to that of the creeping organisms would ever enable anything to swim as quickly as bacteria. Migula (1897, p. 110) is of the same opinion.

7. In fig. 34 of his paper Pijper (1946) gives a sketch of a typhoid bacillus moving forward with its axis perpendicular to the direction in which it swims. Typhoid bacilli often do swim in this way, but it is impossible that a bacterium moving forward by a spiral motion of its body should be able to do so. With flagella as organs of locomotion, however, this movement is quite possible.

8. Pijper (1946, p. 334) writes:—"The argument that it is still the tail that provides the motion and, in doing so, makes the body rotate and undulate, does not bear analysis. The thin feeble tail can hardly have enough power to bend and curve the rigid cell wall to the extent seen and photographed by me". I can agree with Pijper that flagella will never have power enough to bend a bacterium such as he has photographed, but this is no argument that flagella are not organs of locomotion or are perhaps too weak for this purpose. However thin flagella may be, they are—either alone or combined in a tail—mechanically rigid enough to propel a bacterium. Their behaviour when shot off shows clearly that they are very rigid. In the dark field one may see how the lost flagellum can be brought into motion by the Brownian movement of a small particle. If a bend of the tail is hit by a particle, the force may be great enough to make the flagellum rotate, so that it moves over a short distance like a small spirillum. The way in which a flagellum is pushed aside by swimming bacteria also shows that they are rigid. If Pijper had been right in saying that flagella are only slime tails, we should not



expect such rigidity. This observation, therefore, also disproves Pijper's theory. I think that these thin threads are much stronger than one might suppose. I once tried to cut with a fine glass needle the sub-microscopical threads of *Hyphomicrobium*, thinking that this could easily be done, but it proved very difficult.

I also cannot see why the bend observed by Pijper must needs be caused by the direct force of the flagella, since non-motile bacteria also are often bent. If a bend is present and we admit that the flagella have not power enough to bend the bacterium, we cannot therefore say that flagella are not organs of locomotion. It is quite possible that the force of the flagella has a more indirect influence, their motion exerting a constant stress on the body of the bacterium so that it grows out curved. With polar flagella rotation is much more rapid than with peritrichous, and the stress exerted on the body will be greater. Further, the force of polar flagella is applied only to the end of the bacterium; that of peritrichous flagella is more equally distributed over the body. If it is true that the strain exerted by polar flagella is the greater, this might explain the frequent association of polar flagella and bent forms—vibrios and spirilla—and would support my view that we must distinguish polar from peritrichous flagella, an idea disputed by Pijper and by Pietschmann.

It is clear that not one of Pijper's arguments can be reconciled with my observations and his ideas are also opposed by the following considerations:—

1. On Pijper's theory it is impossible to explain the swimming motion of sarcinæ such as *Sarcina ureæ*, which swims very well. Such a packet of bacteria can swim only by means of flagella. The same remark is made by Conn and Elrod.

2. The lively movements of *Hyphomicrobium vulgare* when it tries to free itself from the mother cell is possible only with the aid of flagella.

3. The same may be said of *Sphacrotilus natans*, which also makes lively movements when it tries to escape from the end of the sheath. The body of this organism (fig. 21) seems to me quite unsuited to the making of spiral movements and this is true of all coccoid bacteria.

4. It is well known that only motile typhoid bacilli have H antigen, which is located in the flagella or, according to Pijper, in the slimy capsule. This argument would lead to the strange conclusion that bacteria encumbered with a capsule are motile and that only those without capsules are non-motile. In my opinion there cannot be the least doubt that bacterial flagella are organs of locomotion.

#### FLAGELLA AND TAXONOMY

It remains of interest to consider whether the type of flagellation should be regarded as an important characteristic for bacterial

taxonomy as proposed by Migula and others. Pijper was the first to doubt if there really are bacteria with peritrichous flagella; later Pietschmann also expressed her doubts. Pijper observed that bacteria in the dark field possessed far fewer flagella than in stained preparations. Pijper rejected Neumann's explanation of this difference and set in its place the view that flagellar stains do not give a true picture of bacterial flagellation. Pijper (1930) wrote (translated from the German):—"The different results are only ascribable to the capriciousness of the staining method if not to the faulty technique. One may also be quite ungracious and say that only slime threads are stained". Since the question of the slime threads has already been dealt with, we may now consider if flagellar stains are so capricious as to lack all value.

Four factors at least influence the results of staining—the skill of the investigator, the organism studied, the culture medium on which the organism was grown and the staining method. Of these, the first is perhaps the most and the last the least important. The importance of skill is shown by the repeated failure of students to stain flagella although their teacher has no difficulty in demonstrating flagella at the same time and with the same suspension. Further, one usually has success with a new formula only after a number of trials; first attempts to stain an unknown bacterium are often a failure. Another point is that as a rule flagella can be clearly seen only in a rather small part of the preparation. The different colours of the stained bacteria show clearly that during the staining process conditions are not everywhere alike, and since many flagella are torn off during drying, we need not wonder that in most cases only a few bacteria are successfully stained. Notwithstanding all this, it is certain that, when one has had some experience with the staining technique, the results are so consistent that there will never be any confusion between bacteria with true polar flagella, such, for example, as *Pseudomonas fluorescens* or *Vibrio cholerae*, and peritrichous bacteria like *Proteus mirabilis* and *Salm. typhi*. I therefore consider flagellar staining to be a reliable procedure. I also think it curious that the flagella shown so profusely should be regarded as artefacts. In his paper of 1930 Pijper writes that many of the peritrichous bacteria are "showing off" with flagella lost by others. Although this did not seem very probable, since the arrangement of the flagella is much too neat (figs. 5, 6, 9, 10 and 11), I have done some experiments to test this suggestion. I mixed *Proteus mirabilis* with El Tor vibrio, *Spirillum serpens* and an unknown peritrichous bacterium from swine manure which was poorly flagellated compared with *Proteus*. I could find no evidence that any of these bacteria attracted to themselves any of the numerous detached flagella present in the preparation, not even when these bacteria lay in the neighbourhood of and in the same direction as a strongly flagellated *Proteus* (figs. 5, 6, 7, 8 and 10). In fig. 7 a spirillum is seen in contact with *Proteus mirabilis*, but

even so the spirillum has only polar flagella. Since it was possible that the bacteria mixed with *Proteus* had an opposite electrical charge to that of *Proteus*, and were therefore unable to borrow the *Proteus*'s flagella, I did electrophoresis experiments which showed that all bacteria had a negative charge. All things considered, I think that flagellar staining methods give a true picture of the reality, and that the contradiction between what is seen in stained preparations and in the dark field is only apparent. As Neumann observed, the flagella of *Proteus* are visible in the dark field only when the bacteria are swimming in a gum arabic solution and not in water. He suggested, therefore, that the flagella become visible because many of the primary flagella form a "Zopf" or "plait",\* thus creating a stronger structure better adapted to propel a bacterium through a viscous medium. To test this suggestion he made stained preparations of *Proteus* swimming in water and in a 2-3 per cent. gum arabic solution and showed clearly that the bacteria in water had many more flagella than those suspended in the gum arabic solution, in which case the picture was the same as that of the bacteria in dark field. I repeated these experiments, and photographs of *Proteus mirabilis* (compare figs. 6, 8, 10 and 11 with figs. 9 and 12) and *Spirillum serpens* (compare figs. 13 and 14) clearly confirm the tests of Neumann.

Pijper, however, was not convinced and was of opinion that the flagella became visible because they were covered with a layer of gum arabic. This does not seem probable, however, because if this were true one would expect the thickness of the flagella to be the same over their whole length. But both the dark-field and stained preparations show clearly that the tails are much thicker at the base than at the tip. This must needs be so if the tails consist of many flagella fixed at different points on the body and therefore a little separated at the base. If the flagella are not of the same length the tail will be thinner at the end. Migula (1897, p. 120) has made the same observation. Pietschmann also is of the opinion that Pijper is not right about this. To investigate Pijper's suggestion of the layer of gum, I made the same experiments as Reichert to render the flagella visible in the dark field. I made preparations of *Proteus*, *Spirillum* and typhoid bacilli on coverglasses, under which I ran a drop of Peppler mordant, a silver ethylamine solution or a 5 per cent. gum arabic solution. The preparations were sealed with a rim of vaseline. In not one case could flagella be seen immediately after the preparation was ready, but after 5-10 minutes they were clearly visible in the Peppler and silver ethylamine solutions but not in the 5 per cent. gum arabic solution. Figs. 22, 24, 25, 26, 29 and 31 show the results

\* It may be observed here that the term "Zopf" or "plait" is inaccurate, since the flagella are not plaited, nor are they twined like a cord. They fit easily together because of their spiral shape, as is shown in fig. 36, which is of what the Germans call a "Riesenzopf". Therefore it would be better simply to speak of a tail, a name which does not suggest a special structure and is simpler than "secondary flagellum" or "combined flagella".

## BACTERIAL FLAGELLA



FIG. 1.—*Proteus mirabilis*. Moving train of cells with flagella visible only at the rear end. Exposure  $1/5$  sec.  $\times 1000$ .

FIG. 2.—*Spirillum serpens*. Moving flagella near arrow.  $\times 1000$ .

FIG. 3.—*Salmonella typhi*.  $\times 1000$ .

FIG. 4.—*Proteus mirabilis*.  $\times 1000$ .





## PLATE XLI

FIG. 5.—*Proteus mirabilis* and *Spirillum serpens*, showing separation of polar but not peritrichous flagella.  $\times 1000$ .

FIG. 6.—*Proteus mirabilis* and El Tor vibrio.  $\times 500$ .

FIG. 7.—*Proteus mirabilis* and *Spirillum serpens*.  $\times 1000$ .

FIG. 8.—*Proteus mirabilis* and Bacterium.  $\times 1000$ .

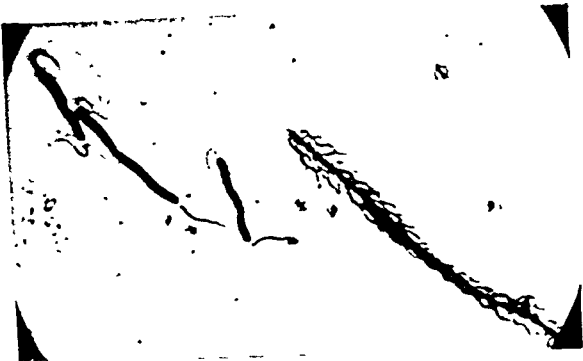
FIG. 9.—*Proteus mirabilis* in 2 per cent. gum arabic solution.  $\times 1000$ .

FIG. 10.—*Proteus mirabilis* and Bacterium.  $\times 1000$ .

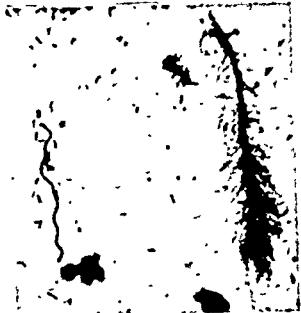
FIG. 11.—*Proteus mirabilis* in water.  $\times 1000$ .

FIG. 12.—*Proteus mirabilis* in 2 per cent. gum arabic solution.  $\times 1000$ .

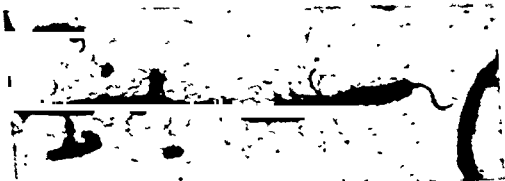
BACTERIAL FLAGELLA



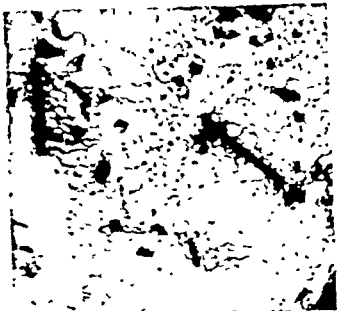
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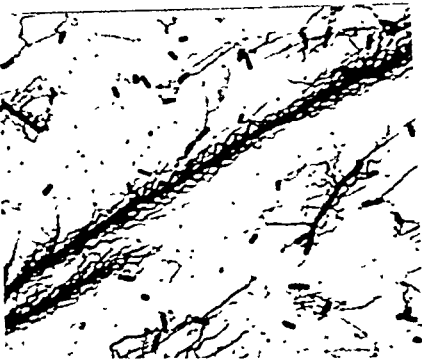
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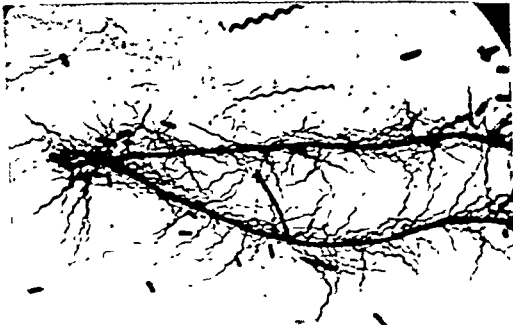
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PLATE XLII

FIG. 13.—*Spirillum serpens* in water.  $\times 1000$ .

FIG. 14.—*Spirillum serpens* in 10 per cent. gum arabic solution.  $\times 1000$ .

FIG. 15.—Bacterium in water.  $\times 1000$ .

FIG. 16.—Bacterium in 2 per cent. gum arabic solution.  $\times 1000$ .

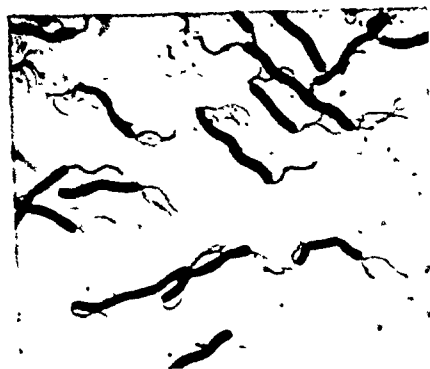
FIG. 17.—*Spirillum* with unfolding tail.  $\times 1000$ .

FIG. 18.—*Salmonella typhi*.  $\times 1000$ .

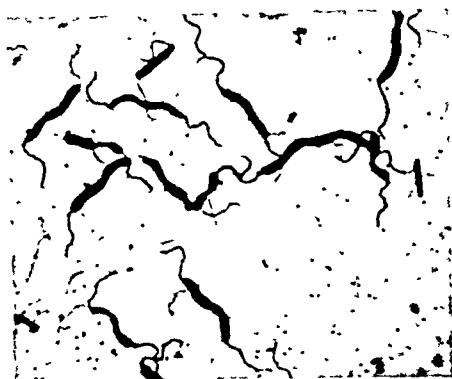
FIG. 19.—*Salmonella typhi*.  $\times 1000$ .

FIG. 20.—*Salmonella typhi*.  $\times 1000$ .

BACTERIAL FLAGELLA



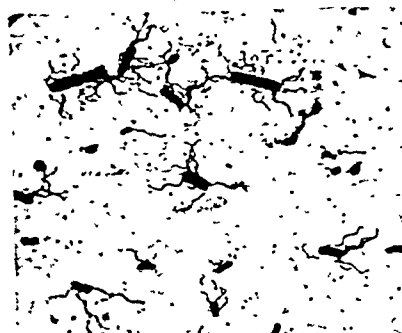
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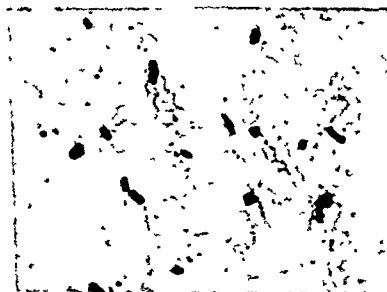
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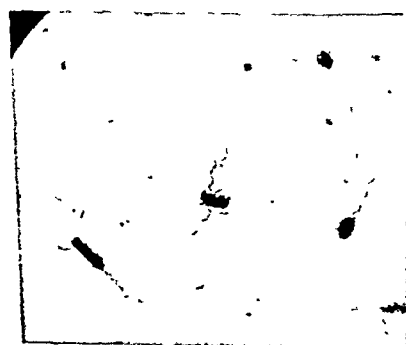
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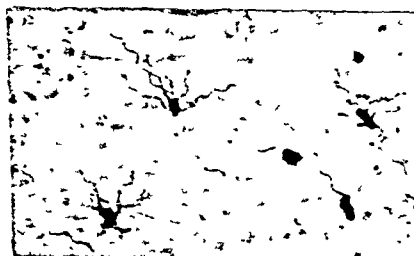
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PLATE XLIII

FIG. 21.—*Spharotilus natans*. Peppler stain (preparation by W. Kauffmann).  
× 1500.

FIG. 22.—*Proteus mirabilis* in Peppler mordant. × 1000.

FIG. 23.—*Proteus mirabilis* in 5 per cent. gum arabic solution. × 1000.

FIG. 24.—*Proteus mirabilis* in Peppler mordant. × 1000.

FIG. 25.—*Salmonella typhi* in silver ethylamine solution. × 1000.

FIG. 26.—*Salmonella typhi* in Peppler mordant. × 1000.

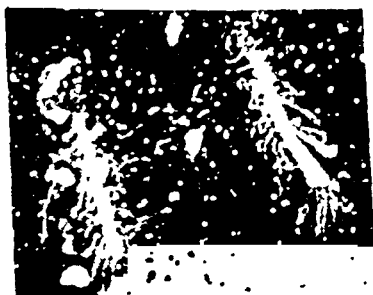
BACTERIAL FLAGELLA



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PLATE XLIV

FIG. 27.—*Hyphomicrobium vulgare*. Stalks and flagella.  $\times 1700$ .

FIG. 28.—*Proteus mirabilis* in silver ethylamine solution, partly darkened by irradiation.  $\times 1000$ .

FIG. 29.—*Proteus mirabilis* in silver ethylamine solution.  $\times 1000$ .

FIG. 30.—*Proteus mirabilis* in silver ethylamine solution, totally darkened by irradiation.  $\times 1000$ .

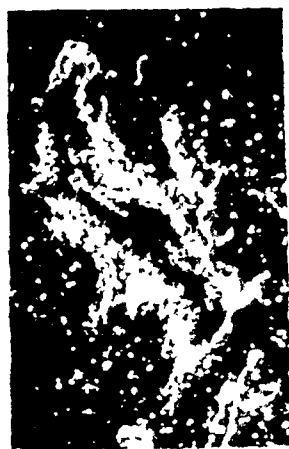
FIG. 31.—*Proteus mirabilis* in silver ethylamine solution.  $\times 1000$ .

FIG. 32.—As fig. 30, but photographed in transmitted light.  $\times 1000$ .

BACTERIAL FLAGELLA



27



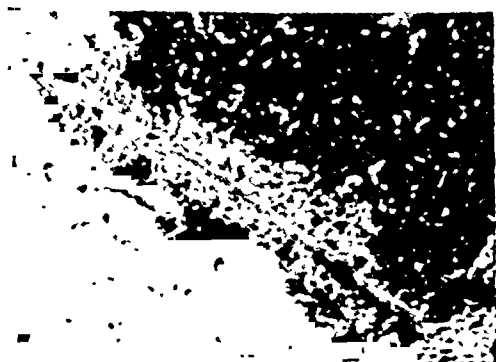
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31



32





## PLATE XLV

- FIG. 33.—*Proteus mirabilis* in India ink. Fields around stationary bacteria swept clear by flagellar movement.  $\times 1000$ .
- FIG. 34.—*Proteus mirabilis*.  $\times 5000$ .
- FIG. 35.—*Proteus mirabilis*.  $\times 2500$ .
- FIG. 36.—A typical structure formed by a collection of flagella.  $\times 1500$ .
- FIG. 37.—*Acetobacter*.  $\times 2500$ .
- FIG. 38.—*Acetobacter*.  $\times 2500$ .
- FIG. 39.—*Proteus mirabilis* in silver ethylamine solution, darkened by irradiation and photographed in transmitted light.  $\times 1000$ .

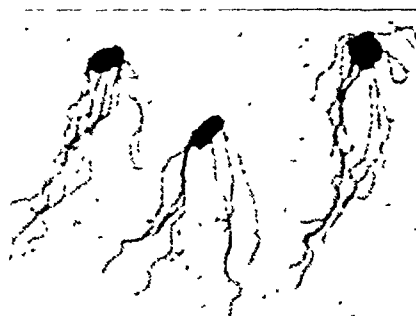
BACTERIAL FLAGELLA



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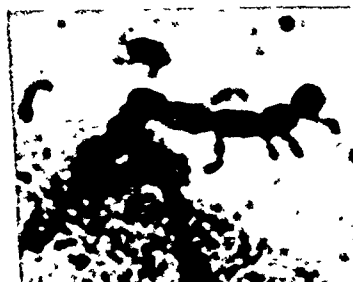
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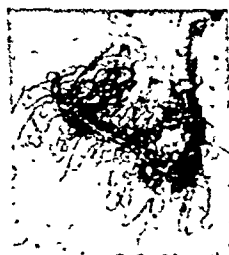
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39



BACTERIAL FLAGELLA

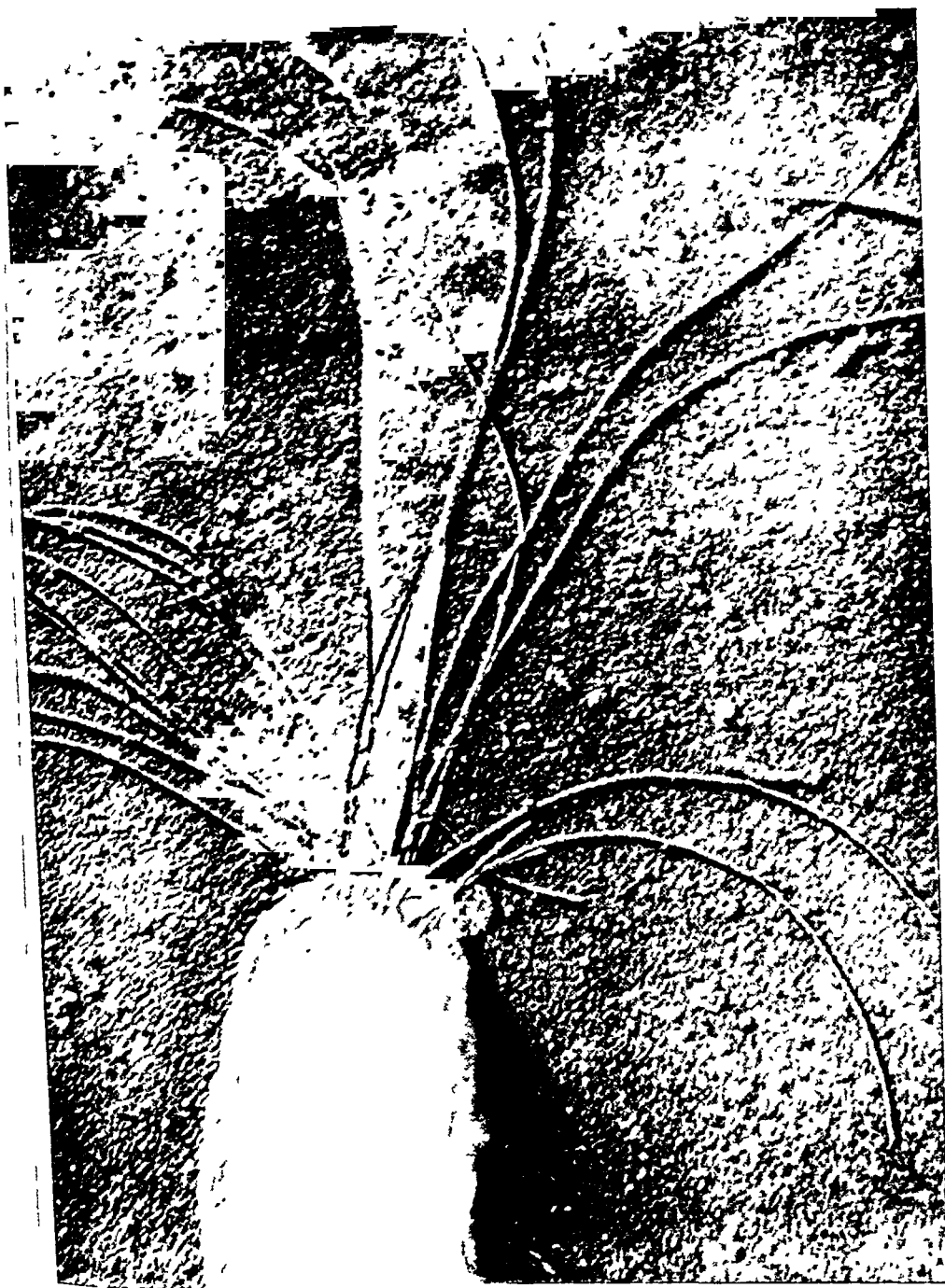


FIG. 40 — *Spirillum serpens*  $\times 30,000$  (Electron photomicrograph by Miss W. van Iterson, Institute for Electron Micro-copy, Delft.)





BACTERIAL FLAGELLA

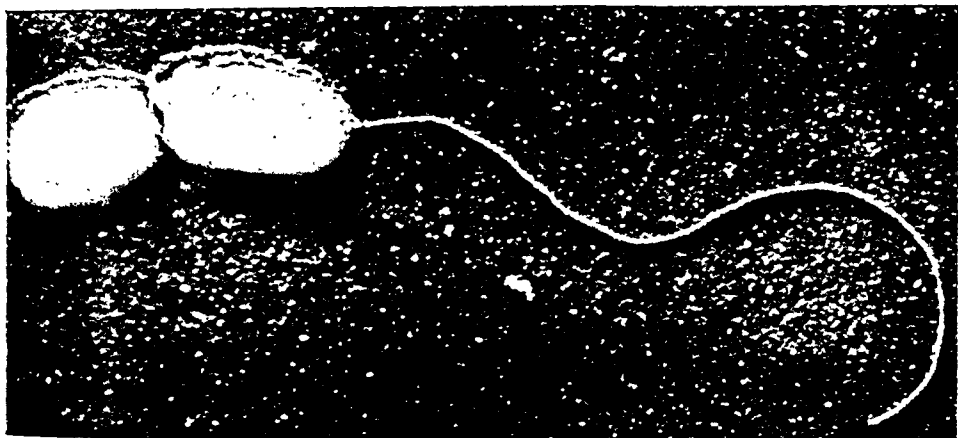


FIG. 41.—*Vibrio metschnikovii*.  $\times 20,000$ . (Miss W. van Iterson.)



FIG. 42.—*Spirillum serpens*.  $\times 22,500$ . (Miss W. van Iterson.)

may stick together, because she observed that the flagella of one cell were more difficult to see than those of a few cells swimming together as one unit. Since these different cells can work together, I think that a single cell must certainly be capable of letting its flagella work together. From the foregoing it will be clear that there is no reason why peritrichous flagellation as shown by flagellar staining methods should not be accepted as a real fact.

It remains to be considered if the type of flagellation has any value for purposes of systematic classification. I admit that it is often very difficult to decide whether flagella are peritrichous or polar. One should keep in mind, however, that the division into peritrichous and polar is artificial, and it is quite possible that there are types of flagellation which are intermediate between true peritrichous and true polar—as, for example, the sub-polar. Notwithstanding this, I think that the type of flagellation is of value in bacterial taxonomy. A glance at the scheme of the phylogeny of bacteria by Kluyver and van Niel (1936) and by Stanier and van Niel (1941) immediately shows that one line contains all the bacteria with polar flagella and another line the bacteria with peritrichous flagella. If the flagellar stains were really as unreliable as some investigators suppose, this simplicity would be impossible. Conn and Elrod agree that type of flagellar arrangement is of taxonomic value, though sometimes the exact type is difficult to determine.

### SUMMARY

Flagella are the true organs of locomotion of the rigid bodies of bacteria and not, as Pijper asserts, useless slime appendages.

There is no reason to suppose that peritrichous flagella as demonstrated by flagellar staining are artefacts. Hence the distinction between peritrichous and polar flagella may still be considered a characteristic of systematic value.

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### Appendix

When I made photographs of the silver-stained preparations of *Proteus*, I observed that the appearance of the bacteria after they had been irradiated for 10 minutes had quite changed (figs. 28, 30, 31, 32 and 39). It appears that the stained flagella are light-sensitive. When they are first brought into the dark field they radiate strongly, but after about 10 minutes they begin to darken from the centre outwards (fig. 28), and after some time the whole bacterium appears black. It looks as if the silver is first deposited in the form of a salt and then reduced to black silver. I observed this only with *Proteus* and it seems probable that the flagella of *Proteus* contain a substance which is easily oxidised. Perhaps it is the presence of this substance which makes *Proteus* more sensitive to light than the other bacteria. From the fact that the stained flagella of *Proteus* present an appearance quite different from that of the typhoid flagella, it may perhaps be concluded that they have a different composition. The irradiated silver-stained *Proteus* bacteria are also quite visible with transmitted light, as is shown by figs. 32 and 39. The Peppler tannin preparations give too slight a contrast to be visible in the light field.



## EXPERIMENTAL PULMONARY ARTERIOSCLEROSIS

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(PLATES XLVIII-LI)

WHILE studying sections of human lungs from patients who had died of primary pulmonary hypertension, numerous vascular lesions were encountered which superficially resembled arteriosclerosis\* but showed features suggesting that they might have been the end stage of an organising thrombosis. This diagnostic difficulty was greatly accentuated by the recent work of Duguid (1946) in which he suggested that what is ordinarily regarded as coronary atheroma is, in fact, largely the result of organised thrombosis. It seemed, therefore, that before attempting any further study of the spontaneous human lesions it was necessary to ascertain experimentally how far organised thrombosis in previously healthy vessels could mimic arteriosclerosis. This was attempted by giving repeated intravenous injections of broken-up blood clot in the hope that these would become impacted in the smaller pulmonary arteries without killing the animal and there undergo organisation.

Rabbits were used chiefly because their large ear veins permit repeated intravenous injections. Ideally each animal should have been injected with its own blood clot. This, however, was not thought to be feasible on account of the damage to ear veins which the necessary bleeding and re-injection would have entailed, so human blood was used.

## TECHNIQUE

Ten to fifteen ml. of human blood were collected aseptically (always from the same individual) and allowed to clot in a 20-ml. screw-capped container. When the clot had retracted the serum was poured off and the clot was shaken hard by hand with sterile glass beads of 5 mm. diameter for about 10-15 minutes. This reduced the clot to fragments which, when floating, appeared to be about 1-2 mm. in diameter. This mass of broken clot was then washed repeatedly with sterile saline until the sedimented fragments of fibrin were buff to pink in colour. It was hoped that by thus getting rid of serum and red cells it would

\* The arterial lesions discussed in this paper were mostly devoid of fat and the terms atheroma or atherosclerosis are not properly applicable. The general term arteriosclerosis has, therefore, been used for lack of a better.

be possible to minimise the risk of allergic reactions. The broken fibrin was then suspended in a volume of saline equal to the original volume of blood and the suspension injected intravenously. Seven rabbits were used to ascertain the optimum dosage. These animals were given one or two injections of 0.25 to 2.0 ml. of suspension of clot and were all killed within 24 hours. A dose of 1 ml. proved to be satisfactory and was well tolerated, but was found on section of the lungs to give rise to very few emboli. It was therefore necessary, in order to produce an adequate number of lesions for study, to give repeated doses. These seven animals, being healthy, apart from occasional embolism causing symptoms of a few hours' duration, were used as controls for the study of normal rabbit pulmonary arteries and for right ventricular size.

Ten rabbits were used for the main experiment. They received injections twice weekly for three weeks. They were then given a rest, mainly to let their ear veins recover, and a second series of injections was started. Five of these animals died in the course of these injections, which were then discontinued, and the remaining five rabbits were killed at intervals to ascertain the late results of organisation of the emboli. The details of the experiment are set out in the table. When an animal died or was killed the thoracic contents were removed and the lungs were distended via the trachea to their normal size with formol-saline and the whole placed in formol-saline to fix. Blocks were taken from various parts of all lobes and paraffin sections cut. At least one block from each animal was cut frozen and stained for fat.

## RESULTS

### *Control series*

Impacted emboli were scanty and usually only one or two were found in several blocks. The pulmonary arteries were otherwise all healthy. The intima was of minimal thickness and lay as a rule on a single well-defined elastic lamina. In an occasional artery the internal elastic lamina was two or even three layers thick, but this was exceptional and was never sufficient to cause any appreciable intimal thickening.

### *Experimental series*

Significant arterial lesions were found in all 10 animals. Owing to the fact that repeated doses of clot were given it was not possible to estimate with any accuracy the age of the lesions studied, but it was possible to find all stages of development from recently impacted clot up to completely healed lesions. The impacted clots (fig. 1) consisted of tight masses of fibrin containing a few leucocytes and red cells. All the early emboli were of this type and, since no ordinary clots were found, it is presumed that propagation of thrombus did not occur. Many of these emboli soon appeared to shrink so that they remained attached to one wall, leaving an eccentric channel, and were soon covered by a layer of endothelium. The first effect of the presence of the embolus appeared to be a brisk cellular reaction by polymorphonuclears and macrophages. This infiltration involved the whole wall of the vessel and formed a cuff outside the adventitia (fig. 2). The polymorphonuclears were transitory and soon the cell

reaction consisted chiefly of macrophages, fibroblasts and some plasma cells. By this stage the cellular infiltration was largely or completely confined to the intima and clot, leaving the media and adventitia apparently normal (fig. 3). The next stage in the organisation was the apparently rapid replacement of the clot by collagen and coincident with this there was an increase of elastic in the form of a splitting off of new layers (fig. 4). After this stage of fibrous replacement and disappearance of fibrin the lesions underwent a slow and steady process of consolidation (fig. 5). This took the form of a shrinkage of the intimal thickening, with opening up of the lumen and an increase in the production of elastic tissue so that the lesions contained more and more fine elastic fibres and relatively less and less collagen: they were by this time virtually acellular (figs. 6 and 7). This process of elastic hyperplasia was apparently complete in about 25 days, because no vessels with only fibrous intimal thickening were found in animals in which the last dose was 25 days or more previously. In the four animals which were allowed to live for several months after the last injection, the lesions were strikingly less severe. In fact, they rarely consisted of more than a minor degree of elastic intimal thickening, usually affecting only a part of the circumference of the vessel (fig. 8).

The lesions in all 10 animals were widespread and severe. The vessels most commonly affected were those of about 400-500  $\mu$  external diameter, but the lesions were by no means limited to these and were found quite frequently in smaller arteries of well under 200  $\mu$  (figs. 9 and 10). In spite of the fact that these vessels were originally plugged by emboli (figs. 1 and 2) they all regained their lumina and none was found with an organised occlusion; in fact, by the time organisation was well advanced the effective lumina were but little reduced. In the smaller vessels of about 200  $\mu$  the intimal thickening was often curiously regular (fig. 9). This finding opens up the possibility that such intimal thickening represents not organised emboli but involution due to the parent vessels being partly occluded and the blood flow reduced. Such a possibility cannot be denied but, since the recognisable stages of early organisation were seen in some of these small vessels, it is fair to assume that in these at least the intimal thickening was directly due to organisation of emboli.

Fatty change was looked for in frozen sections in all animals but was only found in one artery of one animal (no. 2). In this case (fig. 11) there were two thin bands of anisotropic fat in a patch of intimal thickening.

*Heart weight.* In all animals the right and left ventricles were separated and weighed according to the method of Herrmann and Wilson (1921-22) and the results expressed as RV/LV per cent. (table). In the seven control animals the mean figure obtained was  $63.7 \pm 9.05$  per cent., in the experimental group  $66.6 \pm 16.3$  per cent. This difference is too slight to indicate any right heart hypertrophy. It is, however,



interesting to note that in the five animals which died during the course of the experiment the mean figure was  $79.6 \pm 12.3$  per cent.,

TABLE

Table showing the number and spacing of injections, the duration of survival and the RV/LV ratios

Rabbit no.																												RV as Percentage of LV	
1	..	..	..		†																							74	
2	..	..	..						†																			71	
3	..	..	..																										75
4	..	..	..																										104
5	..	..	..																										74
6	..	..	..																										51
7	..	..	..																										64
8	..	..	..																										53
9	..	..	..																										55
10	..	..	..																										45
Weeks	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16													

. = Dose

† = Killed or died.

while in the five which were allowed to recover the mean figure was  $53.6 \pm 4.4$  per cent. Although the number of animals is too small to permit of definite conclusions, the difference between these two groups is more than four times its standard error and therefore significant, while the difference between the animals which died and the controls is more than twice its own standard error and therefore probably significant. These findings are in keeping with the belief that embolism of the pulmonary arteries causes temporary cardiac hypertrophy which resolves as the embolised vessels open up and regain nearly their original bore.

## DISCUSSION

The above findings are of interest in two respects. First, they have a bearing on the recent work of Duguid (1946) in which he suggested that a picture indistinguishable from atherosclerosis can be produced in coronary arteries by the organisation of mural thrombi and, second, they offer a possible explanation of some of the published cases of primary cor pulmonale.

Duguid makes a clear distinction between white and red thrombi, pointing out that it is the latter which tend to undergo softening and to liberate fat. In the present work only "white" clots were used to embolise the pulmonary arteries and therefore, in accordance with his view, it is to be expected that the lesions produced would be free from fat.

EXPERIMENTAL PULMONARY ARTERIOSCLEROSIS

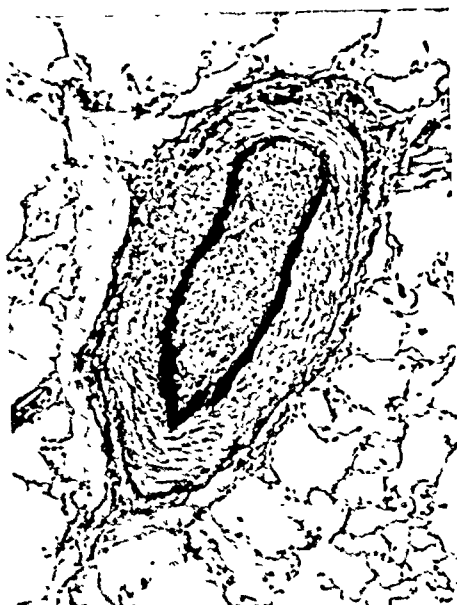


FIG. 1.—Control rabbit killed 5 hrs. after a single injection. Clot impacted in small pulmonary artery of  $250\mu$  diameter. Note normal elastic lamina and absence of reaction.  $\times 112$ .



FIG. 2.—Rabbit 4. Pulmonary artery of  $350\mu$  diameter containing impacted embolus in stage of early organisation, with brisk cellular reaction. Note slit-like lumen.  $\times 100$ .



FIG. 3.—Rabbit 3. Organising embolus in artery of  $500\mu$  diameter. Clot almost entirely fibrosed. Lumen well opened up and lined by rather prominent endothelium. Cellular reaction has largely disappeared. Note that the elastic lamina is still normal.  $\times 100$ .

All sections stained with Lawson's modification of Weigert's elastic stain followed by celestin blue and Mayer's hæmalum and van Gieson.



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## EXPERIMENTAL PULMONARY ARTERIOSCLEROSIS



FIG. 4.—Rabbit 2. Organising embolus in an artery of  $400\ \mu$  diameter. Slightly later stage than fig. 3. Cellular reaction has now totally disappeared and elastic proliferation is commencing.  $\times 100$ .

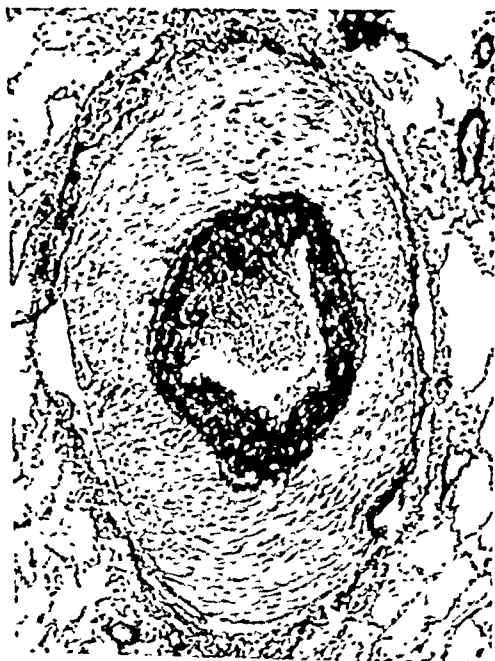


FIG. 5.—Rabbit 1. Late organisation of embolus in an artery of  $560\ \mu$  diameter. Later stage than fig. 4. Note the striking elastic hyperplasia.  $\times 80$ .

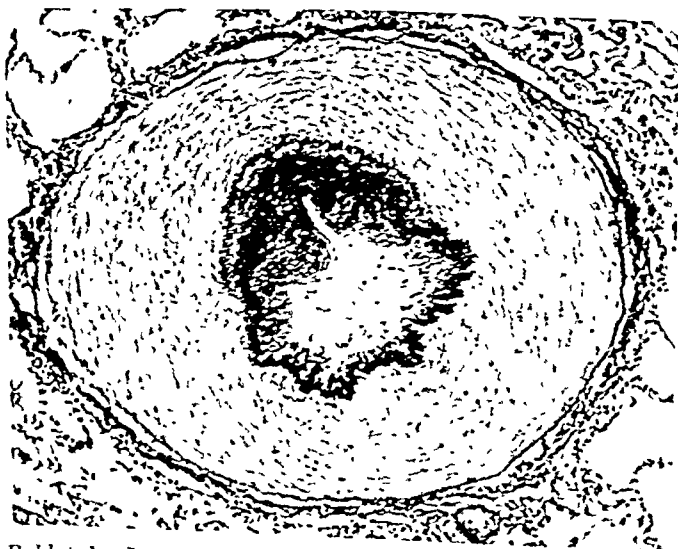


FIG. 6.—Rabbit 1. Late stage of organisation, with massive elastic hyperplasia in an artery of  $600\ \mu$  diameter.  $\times 100$ .





EXPERIMENTAL PULMONARY ARTERIOSCLEROSIS

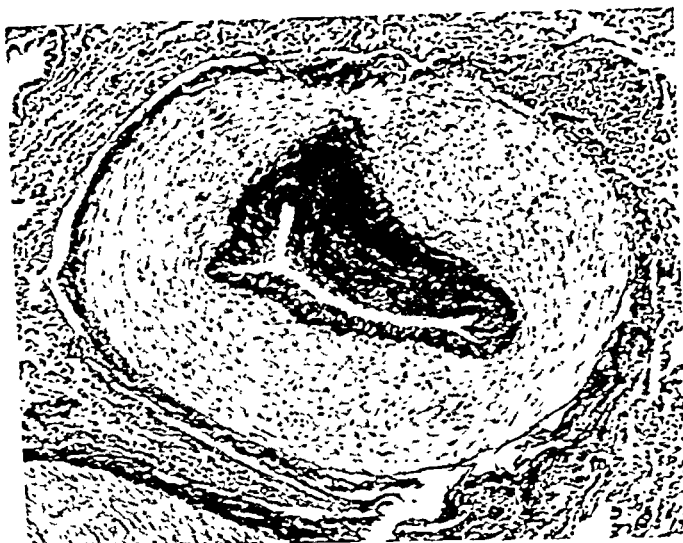


FIG. 7.—Rabbit 1. Late stage of organisation in a large artery of  $660\ \mu$  diameter, showing gross elastic hyperplasia.  $\times 75$ .



FIG. 8.—Rabbit 9. Seventeen weeks after last injection. Late stage of organisation, with shrinkage of intimal elastic thickening in an artery of  $440\ \mu$  diameter  $\times 95$ .



FIG. 9.—Rabbit 9. Late lesion in a small artery of  $180\ \mu$  diameter, showing fibro-elastic intimal thickening.  $\times 205$ .



EXPERIMENTAL PULMONARY ARTERIOSCLEROSIS



FIG. 10.—Rabbit 1. Low-power view of lung showing widespread lesions in arteries of various sizes.  $\times 9$ .



FIG. 11.—Rabbit 2. Frozen section of large pulmonary artery stained with Sudan, showing two streaks of fatty change in the thickened intima.  $\times 45$ .



Among the published cases of primary cor pulmonale, those of Frothingham (1929), Means and Mallory (1931-32) and Castleman and Bland (1946) are believed by the authors to be due to thrombosis of pulmonary arteries. In the light of the present work it is possible that in some at least of the other cases the sclerosis of the pulmonary arteries may have resulted merely from organisation of thrombi or emboli. In the cases of Rosenthal (1930, cases 2 and 3), Waring and Black (1934), Seely (1938), East (1940, case 3), Boyd (1944) and Gilmour and Evans (1946), there are illustrations of lesions which can be almost exactly duplicated by those of the present experiment. It is of course unsafe to apply to human disease the results of animal experiment and it would be quite unjustified to suggest that the cases listed are, in fact, examples of organisation of thrombi. Nevertheless, if emboli can heal so completely and leave behind only fibro-elastic intimal thickening in a vessel with a clear lumen, it becomes necessary to consider carefully the possibility of multiple small emboli in any case of pulmonary arteriosclerosis, even though organising emboli are not readily apparent.

#### SUMMARY

Rabbits were given a course of intravenous injections of finely fragmented fibrin clot which produced multiple small pulmonary emboli. These were rapidly organised and the lumina of the vessels reconstituted.

The vessels in which such lesions had healed were left with fibro-elastic intimal thickening which was morphologically indistinguishable from spontaneous arteriosclerosis.

It is suggested that some cases of primary pulmonary arteriosclerosis having this histological appearance may be the result of healed pulmonary embolism.

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# THE LARGE-CELL SMALL-ACINAR THYROID TUMOUR OF LANGHANS AND THE INCIDENCE OF RELATED CELL GROUPS IN THE HUMAN THYROID

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(PLATES LII-LV)

LANGHANS, in 1907, in a classic article on the malignant epithelial tumours of the thyroid, proposed a classification which has been little altered by many later improvements. He included as one of his groups a "grosszellige kleinalveolare Struma" or "Struma post-branchialis" and described five cases, only two of which were definitely malignant. The condition was re-christened "Hürthle-cell tumour" by Ewing (1919) and, of the fifteen cases since published which are generally regarded as falling within this group, the majority have been so called. The present study deals with two cases apparently representing two distinct conditions which have been confused under this one heading and attempts to relate both to similar cell groups which are common in the human thyroid.

## CASE I

A man aged 42, a signwriter from Shepherd's Bush, had had a lump on the right side of his neck for 15 years. It had increased in size steadily throughout that period, rather more rapidly in recent years. He had had some pain on the same side of the neck for 2 or 3 years, indefinitely related to the lump. His history otherwise was without important events except for a mild attack of rheumatoid arthritis at the age of 37, with good recovery. Examination showed a thin healthy man with a mobile well defined hen's-egg mass in the lower part of the right lobe of the thyroid. There was no indication of thyrotoxicosis. Elsewhere in the body he showed only a moderate congenital asymmetry of the chest and slight residua of arthritis. In August 1947 the tumour was excised with a substantial amount of the normal gland by Mr R. Shackman: recovery was uneventful and now, twelve months later, he is well and without recurrence.

The tumour as received (fig. 1) weighed 35 g. with some attached normal thyroid. It was well defined, ovoid, solid and fleshy, and of an opaque brownish colour on section, with fibrous bands and some points of calcification. Low power microscopy (fig. 2) shows a solid cellular tumour lying between broad trabeculae of hyaline collagen: in most areas these separate the tumour sharply from compressed but otherwise normal thyroid. At higher magnification (fig. 3)



the tumour is strikingly uniform in all areas: it is obviously thyroid of some kind, but there is transition neither to normal gland nor to less well differentiated tumour. Small closely packed acini are seen, often with small lumina containing a little dried up colloid and rarely forming short tubules. The cells are large, averaging  $20\ \mu$  in diameter, very irregular in shape and often very clearly demarcated from each other: the cytoplasm is uniformly cloudy or indistinctly granular. In eosin-stained sections the even opaque pink colour given to whole fields by this uniform cytoplasm is very characteristic. The nuclei vary considerably in size and often possess nucleoli, but they centre on a generally thyroid type. Various stains were tried without detecting any specific reaction of the cells. Methods using acid fuchsin as a cytoplasmic stain (Masson's trichrome, picro-Mallory and Ehrlich's triacid) emphasise a rather indistinct network or honeycomb of the kind seen in fig. 6, and it seems that the distinct granules seen often with eosin and constantly with phosphotungstic acid-haematoxylin represent the nodes of this network. This pattern seems in any case to be merely an exaggeration of one to be made out by similar methods in normal thyroid epithelium.

There can be no reasonable doubt that this tumour is to be grouped with those described by Langhans. An exact assessment of its malignancy is not so easy. In most areas its capsule is formed by thick hyaline collagen, mitoses are rare and no muscle-walled vessels are invaded. On the other hand at several points the capsule seems to be broken through (e.g. fig. 2), apparently by infiltration of capillaries. But it is considered that provided he can be watched carefully no further treatment is indicated.

## CASE II

A spinster aged 51, a dressmaker from Henley, had had a lump on the right side of her neck for eight years. During the middle four of these years it seems almost to have disappeared, but recently it had begun to cause some dysphagia. Her complaints were many and her history difficult to assess. She had some tremor and slight exophthalmos and was considered to have, or to have had, some degree of thyrotoxicosis, though shortly before operation her basal metabolic rate on two occasions was +1 and -1. The tumour was excised by Mr A. K. Henry, who was able to shell it out with comparative ease; recovery was uneventful. No further treatment was given and eight years later the patient had no relevant complaint.

The tumour was of the size and shape of a tangerine orange, externally smooth, and weighed 50 g. Under the low power (fig. 4) it shows a smooth capsule, a peripheral vesicular zone resembling normal thyroid at this magnification and a central solid area resembling the tumour of case I. A higher power (fig. 5) shows this resemblance to be extremely close: it shows, in addition, that most of the cells lining the larger acini of the peripheral zone are large, granular and eosinophilic: the zone in fact forms a transition, absent in case I, between tumour and normal thyroid. There is no evidence of secretory hyperactivity, no lymphocytic infiltration and no indication of malignancy.

It is believed, for reasons which will appear later, that this case is not to be classified with case I in Langhans's group, but represents the self-cure of a toxic adenoma by regressive changes in the active epithelium.

## *Related cells in the human thyroid*

It seems to me impossible to discuss this tumour without paying attention to the occurrence of groups of cells similar to those of the tumour in the human thyroid under various pathological conditions. Accordingly an attempt has there been made to assess the incidence

of these cells. They are surprisingly common : in the first 420 thyroids searched for them at least one complete acinus of large pink granular cells was found in 76. The acceptance of smaller collections of cells might have made this figure higher, but in view of the very mixed material studied, purely cytological criteria have been avoided as far as possible.

Very little of these results is wholly new. Though no systematic study seems to have been made, various authors have recorded the occurrence of these cell groups in pathological thyroids. Askanazy (1898) was the first to note them in toxic thyroids: Roussy and Clunet (1914) illustrated them very beautifully in the same condition and Wegelin (1926) was aware of their wider distribution. Womack (1944) mentions the occurrence of "Hürthle cells" in thyroiditis. The epithelial cell changes in this condition have been described often enough, *e.g.* by Shaw and Smith in 1925-26 and Vaux in 1938, but Womack seems to have been the first to use this particular name. Hamperl (1936-37) describes their occurrence (as "oncocytes") in thyrotoxic and senile atrophic thyroids.

For the time being I wish to label these cells "Askanazy cells". The term "Hürthle cell" has been used fairly generally for them, but should, I think, be avoided. On the one hand Hürthle (1894) merely redescribed the parafollicular cells of puppy thyroids which, as he himself acknowledges, had already been demonstrated to the Royal Society by Baber in 1876 and more fully in 1881, and on the other hand the relation of the parafollicular cells to those now under discussion is very doubtful. Nonidez (1931-32, 1932, 1933) and Zechel (1931, 1932, 1933) and Raymond (1932) have made detailed studies of these cells in dogs, cats and rabbits, but a survey of recent descriptions of the human thyroid, orthodox and unorthodox (*e.g.* Williamson and Pearse, 1923; Rienhoff, 1929; Moritz, 1931; Marine, 1932; Goormaghtigh and Thomas, 1934; Means, 1937; Maximow and Bloom, 1938) shows little evidence of support among histologists for the occurrence of any similar structure in the normal human gland. It seems most significant that parafollicular cells are frequent, in those animals in which they occur, only in the first few weeks of life, whereas the cells with which we are here concerned occur chiefly at the opposite end of life. Zechel's (1933) only illustration of human "macrothyrocytes" is in a man of 75.

I have not yet succeeded in applying Nonidez' silver technique (1931-32, 1932) for parafollicular cells to human thyroids containing Askanazy cells. The material must of course be fresh, and suitable surgically excised thyroids are becoming increasingly rare.

#### *Incidence of Askanazy-cell groups*

My material consists chiefly of 250 thyroids resected since 1936 at the Postgraduate Medical School of London, and 150 normal thyroids obtained *post mortem*. Other material has been used for the

illustration of particular conditions, but the graphs and related statistics are based on the unselected series.

*Normals.* Fig. 8 shows the occurrence of Askanazy-cell groups in normal thyroids. It will be noted at once that they are absent in the younger age group and uncommon even in old men, but become very common in older women, especially over the age of 60. "Normal" is, of course, a relative term. I have eliminated cases showing clinical evidence of thyroid disease or more than minor abnormality to the naked eye, but Askanazy cells seem to be especially associated with the residuum of minor abnormalities. In women the

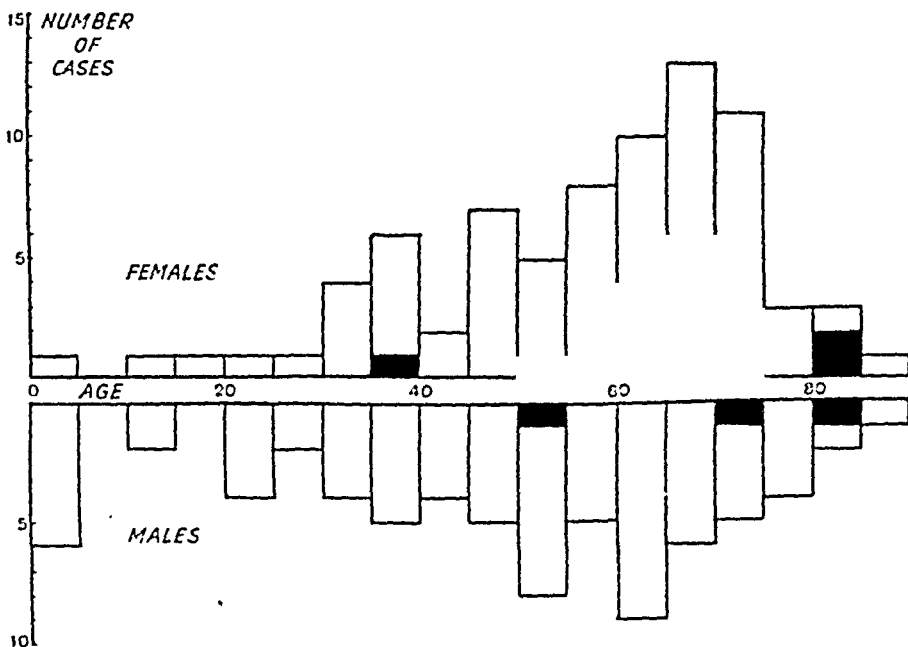


FIG. 8.—Normal thyroids. Results in 150 post-mortems in which no gross lesion was present in the thyroid.

In these graphs the histograms show the number of cases examined in five-year age groups, females above and males below the base line. Blacked-in portions show the number of cases in which Askanazy cells were found.

association with lymphoid hyperplasia is especially close: 78 of the subjects were women, of whom 27 showed lymphocytic infiltration, and of these more than half (15) showed also Askanazy cells. Only three women showed Askanazy cells without lymphocytic infiltration. Nodules of Askanazy cells are often actually embedded in lymphoid tissue (fig. 7) and it is unusual to see them without some surrounding lymphocytic infiltration. The association is much less marked in men, of whom two showed Askanazy cells only, five lymphocytes only and one both.

*Thyrototoxicosis.* Fig. 9 shows the findings in 135 surgically removed thyroids with hyperthyroidism. There is seen to be a moderate (16.3 per cent.) frequency, confined to women and significant enough

in comparison with corresponding age groups of normals. The lower graph shows the much greater frequency (50 per cent.) in toxic adenoma: the group is small but the observation is reinforced by the much greater proportion of positives in the upper graph among glands showing excessive macroscopic nodularity.

The association with lymphoid tissue noted in normal thyroids is maintained. Fig. 10 is a typical nodule from a case of Graves's disease. Fig. 11 shows acini embedded in lymphoid tissue in a toxic adenoma. Fig. 12 is from a remarkable case of a woman of 72 who

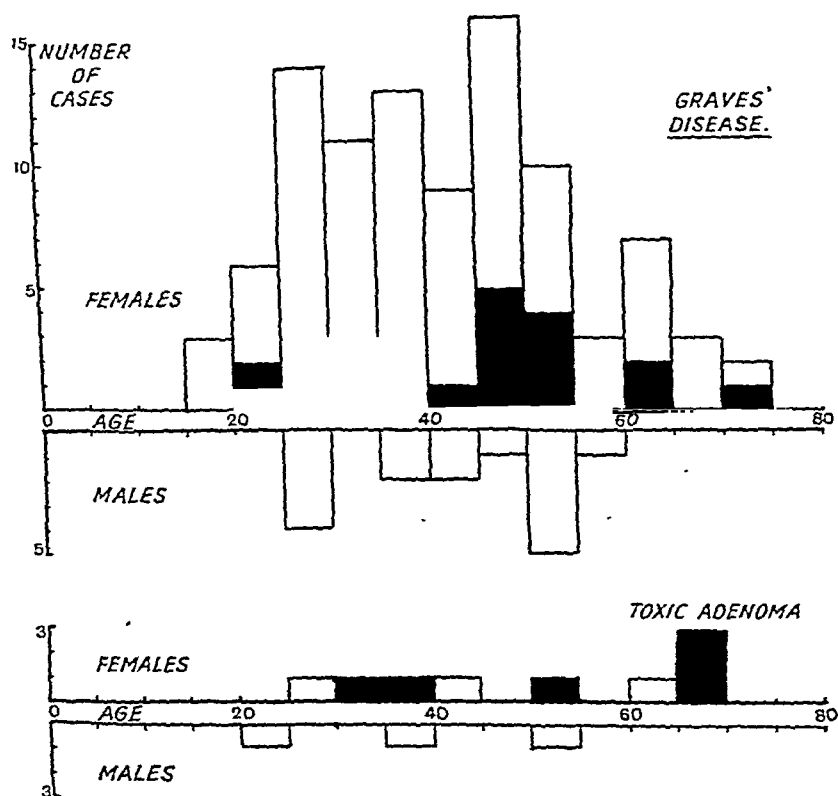


FIG. 9.—Thyrotoxic thyroids. Results in 123 cases of diffuse toxic goitre and 12 of toxic adenomata treated by resection.

died of thyrotoxicosis; the thyroid weighed 375 g., was markedly nodular, and shows secretory hyperactivity histologically, with lymphoid hyperplasia and conversion of roughly a third of the area of each section to the Askanazy type, including several nodules up to 8 mm. in diameter of tissue hardly distinguishable from the tumour of case I.

The great bulk of these cases had received pre-operative iodine, but I have been permitted by Professor Dible to examine a group of pre-iodine Graves's thyroids from his private collection and found approximately the same incidence. Thiouracil also seems to have little effect in this respect. There seemed no obvious correlation

between the occurrence of the cell groups and any clinical feature except sex and nodularity: it was rare in the fulminating toxæmias of younger women but these in any case are usually diffuse in type.

*Non-toxic goitre* (excluding foetal adenoma). Fig. 13 represents a very mixed group of colloid goitres, nodular goitres, simple adenomas and the like. Askanazy cells are infrequent (5 per cent.).

*Foetal adenoma*. Of 11 examples, 4 showed the change: figs. 14 and 15 are examples. Though the affected cells are comparable, so far as can be seen, in all other respects to Askanazy cells arising elsewhere, it is noteworthy that this is the only important class in which they are not associated with excess of lymphocytes.

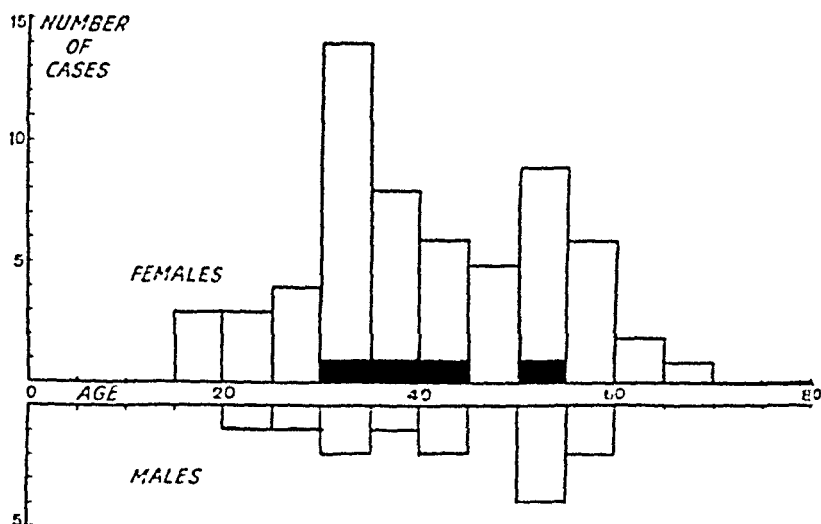


FIG. 13.—Non-toxic goitres. Results in 74 surgically treated cases of thyroid disease, mostly called colloid goitre, nodular goitre or simple adenoma. Carcinoma, foetal adenoma, thyroiditis and toxic lesions are excluded.

*Thyroiditis*. Six cases of Hashimoto's disease have all shown complete or nearly complete absence of normal epithelium, which has been extensively replaced by the Askanazy type of cell (fig. 16). Four cases of Riedel's struma have shown no example of the change. The material is quite inadequate for any significant contribution to this controversial field but the fact invites speculation. Is it possible for instance that the age and sex incidence so relied upon by those who regard the two conditions as distinct (Joll, 1939-40) are merely an expression of an altered reactivity in the thyroids of older women? Perhaps at least one may take the presence of extensive Askanazy cell change in thyroiditis to be of good prognostic import.

*Myxædema* seems (though again six cases provide inadequate material for purposes of comparison) either to show, like Hashimoto's disease, widespread lymphocytic infiltration and Askanazy cells, or, like Riedel's disease, widespread fibrosis and no Askanazy cells.

LANGHANS ADENOMA OF THYROID



FIG. 1.—Case I; the tumour on section.  $\times 1.5$ .



FIG. 2.—Case I. An area of the tumour showing (above left) the hyaline collagen which forms trabeculae and capsule (here partly calcified) and (above right) a nodule of tumour appearing to breach the capsule. Masson's trichrome.  $\times 6$ .

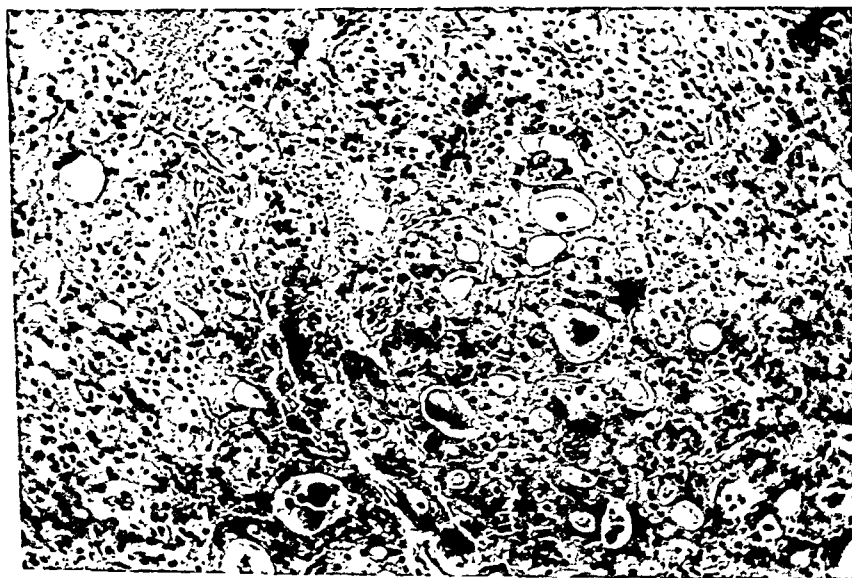


FIG. 3.—Case I. The characteristic homogeneous "large-cell small-acinar" structure. H. and E.  $\times 125$ .



## LANGHANS ADENOMA OF THYROID



FIG. 4.—Case II. Showing the transitional outer zone and the "large-cell small-acinar" inner zone. H. and E.  $\times 4.5$ .



FIG. 5.—Case II. The junction between the zones, with the transitional zone above. H. and E.  $\times 125$ .

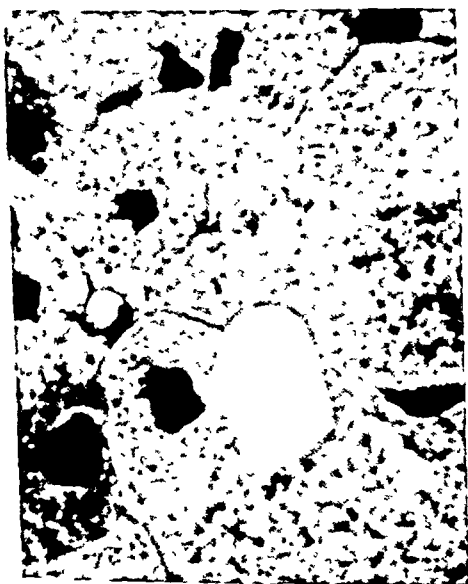


FIG. 6.—Case II. Showing the fuchsinophil network in the cytoplasm, with its deeper-staining nodes. Picro-Mallory.  $\times 960$ .



FIG. 7.—"Normal" thyroid. A nodule of Askanazy cells in the thyroid of a woman of 84 who died of chronic bronchitis and cor pulmonale. H. and E.  $\times 125$ .





LANGHANS ADENOMA OF THYROID



FIG. 10.—Toxic goitre. A typical island of Askanazy cells (below centre). From a woman of 52 with Graves's disease. H. and E.  $\times 125$ .

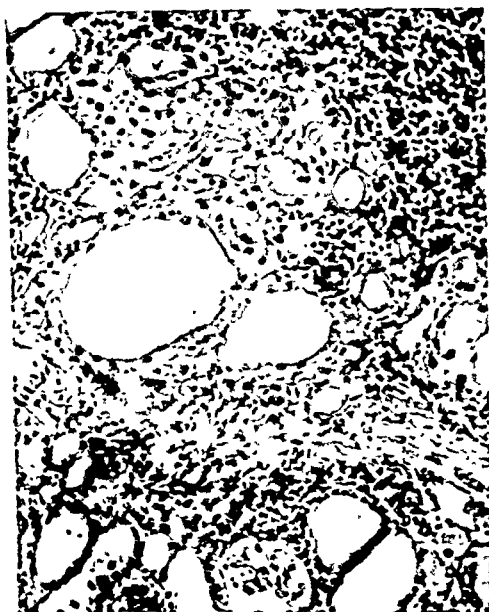


FIG. 11.—Toxic adenoma. Askanazy cells embedded in lymphoid tissue. From a woman of 62. H. and E.  $\times 125$ .



FIG. 12.—Toxic nodular goitre. Extensive Askanazy-cell change. From a woman of 72. H. and E.  $\times 125$ .



LANGHANS ADENOMA OF THYROID



FIG. 14.—Fœtal adenoma. Two Askanazy-cell nodules, that on the right transitional, that on the left typical, in a fœtal adenoma from a woman of 39. H. and E.  $\times 53$ .



FIG. 15.—Fœtal adenoma. Focal change to the Askanazy cell type in a fœtal adenoma from a woman, age unknown. The acini retain something of the parent shape. H. and E.  $\times 125$ .

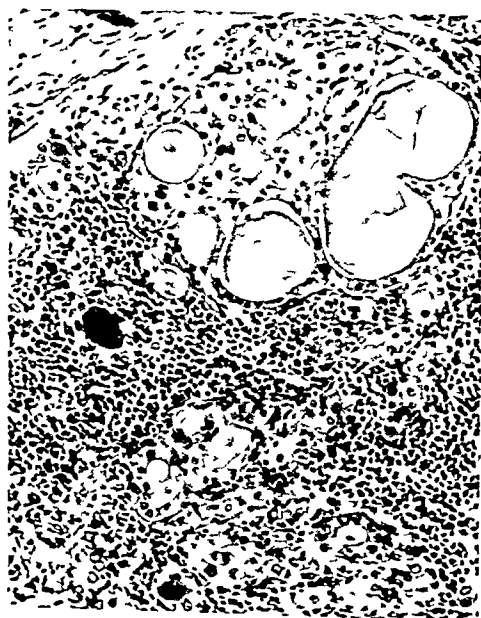


FIG. 16.—Hashimoto's disease in a woman of 67. The upper nodule shows especially clearly the Askanazy type of epithelium. H. and E.  $\times 125$ .



*Miscellaneous conditions.* No other significant association was seen in the remaining material, most of it carcinomata or thyroglossal cysts. Two examples of ordinary carcinoma arising from thyroids with Askanazy cells and lymphocytic infiltration were seen. The fibrosis associated with abscess or secondary tumour does not excite the Askanazy-cell change.

### *Origin of the tumour*

There has been a variety of hypotheses on the origin of this tumour. Langhans himself implies by his alternative title of "*Struma post-branchialis*" belief in an origin from the post-branchial body of Sophia Getzowa (1907, 1911). The share of the fourth branchial cleft in the origin of the mammalian thyroid still seems doubtful (Marine, 1932): the tubules and cell groups described by Getzowa in the thyroids of cretins may well have a pathological rather than an embryological explanation, but in any case this hypothesis has suffered the general loss of repute of attributions of adult tumours to foetal structures. Until a tumour is identified arising from fourth cleft structures outside the thyroid there cannot be said to be any real evidence in its favour.

Eisenberg and Wallerstein's claim (1932) of a parathyroid origin has been very faithfully dealt with by later writers. It was based on the resemblance of the cells of the tumour to the oxyphil cells of the parathyroid. This is part of the more general group of similarities which led Hamperl (1931 *a* and *b*, 1933 and especially 1936-37) to his concept of the oncocyte. Large cells with eosinophilic honeycomb or granular cytoplasm and small or irregular nuclei, with a tendency to increase with age and with no apparent function, have been described in the salivary glands, the parathyroids, much of the respiratory tract (Nothner, 1946), the pancreas, the pituitary, the lachrymal glands, the oesophagus and perhaps the mastitic breast, and in most of these sites can give rise to tumours. It is not difficult to confirm many of these resemblances, but the significance, if any (for the whole may be coincidence), is quite obscure. There is nothing positively against calling Askanazy cells thyroid oncocytes and the Langhans tumour a thyroid oncocytoma, but there is so far little except logistic profit to be gained thereby.

Ewing suggested the origin from Hürthle cells. Until the relation between Hürthle and Askanazy cells has been elucidated this must remain a doubtful attribution.

Wilensky and Kaufman (1938), recognising the mixed histology of their cases, suggested that some unexplained physiological change occurs as a secondary phenomenon in an adenoma or carcinoma of more ordinary type and that the behaviour of the tumour bears no relation to this "Hürthle-cell change" (as they call it), but only to the nature of its precursor. Basically, this in my opinion is the

right answer. But it seems to me that this change can occur in two main ways :—

(i) *By change of cell type in a foetal adenoma.* Such change certainly occurs fairly frequently. Other types of thyroid carcinoma can be traced often enough to foetal adenomata. While hesitating to claim it as the sole method, this seems a reasonable hypothesis to explain the origin of the pure large-cell tumour as described by Langhans. It accounts for the lack of lymphocytes, and the curious stroma of my case I is perhaps some additional evidence.

(ii) *By change of cell type in a toxic adenoma or toxic nodular goitre.* As we have seen, a moderate degree of this change is common. In a case such as that illustrated in fig. 12 there are many nodules of what appear to be typical Langhans tumour tissue till one moves to the hyperactive gland. Case II I believe to represent the completion of the process. It is tempting to bring all the tumours into this group, but any attempt to do so founders on the fact of a practically complete negative correlation in reported cases of "Hürthle-cell tumour" between malignancy and toxicity, as a glance at the table in Morrow (1945) will show.

#### *Reported cases*

In accordance with the above idea, the 22 reported cases (including my own) may be classified into the three following groups.

(I) *The large-cell small-acinar tumour of Langhans proper* (ten cases : mean age 47.6 years), characterised by a uniform cell type, complete absence of toxicity and frequent malignancy.

- (i) Langhans's original five cases (1907).
- (ii) Haagensen's two cases (1931).
- (iii) Martin and Elkin's third case (1939).
- (iv) Harry's case (1941).
- (v) My case I.

Five of these (mean age 53.8, two men) actually metastasised, the other five (mean age 41.4, two men also) were all suspicious. Other cases are mentioned without details by Ewing (1919, two cases), Wegelin (1926) and Clute and Warren (1935), but the condition must still be regarded as rare. Incidentally the last-named authors consider it fairly radiosensitive and in general the prognosis in this disease seems relatively good.

(II) *Toxic adenomata showing extensive Askanazy-cell change.* There are eight fairly certain cases in this group, whose mean age is 46.9 years, and two probables. All are women. Most show a mixed histology and lymphocytosis. None has recurred. All showed some evidence of thyrotoxicosis though this was sometimes equivocal, a finding to be expected if extensive change of this type cures the toxicosis.

- (i) Lobenhoffer's two cases (1909) : (details inadequate but most probably to be included here).

- (ii) Eisenberg and Wallerstein's case (1932).
- (iii) Eberts's case (1933).
- (iv) Wilensky and Kaufman's two cases (1938).
- (v) Martin and Elkin's two cases I and II (1939).
- (vi) Reimann's case (1943).
- (vii) My case II.

In the first case of Martin and Elkin a disappearance of toxic symptoms before operation was associated with a nearly pure cell picture. It seems that here and in my second case all activity has been destroyed by a uniform change in all the active cells. It is noteworthy that in both of these lymphocytes have disappeared also.

(III) *Congenital goitres*. The two cases described by Symmers (1941) and Morrow (1945) are of doubtful relation to the present subject. In both, the whole thyroid at birth was enlarged and uniformly replaced by tissue regarded as similar to that of our tumour. Both seem to have proved curable by operation and radiation, unlike most true neoplasms at that age.

The distinction between groups (I) and (II) does seem to be of some practical significance. Several of the cases in group (II) had been labelled "Hürthle-cell carcinoma" and irradiated, probably quite unnecessarily. Indeed it would seem that a history of thyrotoxicosis negatives malignancy, as it does in thyroid tumours generally (Ward, 1944). A mixed histology and lymphocyte infiltration all point the same way. Only in the absence of thyrotoxicosis, and with a histology uniform throughout the tumour need malignancy be considered: in men it is especially likely.

#### *Physiological significance of the Askanazy-cell change*

Very little can be said about this. There seems to be no good evidence that the cells concerned secrete thyroxin. If a toxic adenoma can be cured by change of this kind, and if the change is also characteristic of Hashimoto's disease (whose subjects are commonly myxœdematous), the cells must be without endocrine function. As to the cause of the alteration I can offer no hypothesis, but it does seem that when not actually tumorous the change is a regressive one. Possibly hyperplastic thyroid acini become ultimately "worked out" and pass through a phase of Askanazy-cell change and lymphocytic infiltration before disappearing.

A quantitative iodine analysis was done on the tumour of case I by Dr Russell Fraser by a modification of Taurog and Chaikoff's (1946) method for blood iodine. The material had been in formalin for some months, but a piece of the normal thyroid adjacent to it, put through as a control, gave a substantially normal figure (over 0.035 per cent.). It therefore seems significant that no iodine could be detected by this very sensitive method in the tumour—additional evidence that cells of this type are non-functional.



## Summary

1. Two cases of the so-called "Hürthle-cell tumour" of the thyroid are described. Case I, that of a man of 42, is a typical example of the "large-cell small-acinar" tumour described by Langhans. This group, which is characterised by uniform histology, absence of toxicity and frequent malignancy, now amounts to ten reported cases. It is suggested that this tumour arises by change of epithelial cell type in foetal adenomata. Case II, that of a woman of 51, is believed to be the result of extensive regressive epithelial changes (of the kind first described by Askanazy) in a toxic adenoma. Half the cases described in the literature as Hürthle-cell tumour are believed to fall into this group.

2. Groups of cells resembling those of the tumours (here called Askanazy cells) are frequent in women over 60, in toxic adenomata, foetal adenomata and Hashimoto's disease. Except in foetal adenomata they are closely associated with lymphocytosis. Men are rarely affected.

3. The physiological significance of the Askanazy-cell change is not clear, but the cells do not secrete thyroxin and their appearance must usually be regarded as a regressive phenomenon. Their relation to the parafollicular cells of puppy thyroids needs further study.

My thanks are due to Professor J. H. Dible, Dr C. V. Harrison and Dr I. Doniach for many suggestions and the loan of slides; to Professor Ian Aird and Mr R. Shackman for permission to use their material; to Dr Russell Fraser for the iodine estimations; to Messrs J. R. Baker and J. J. Griffin for the sections and Mr R. V. Willmott for the photomicrographs.

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# AGGLUTINATION OF COLIFORM BACILLI BY NORMAL RABBIT SERUM

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It is often assumed that the serum of a healthy uninoculated rabbit contains only insignificant amounts of antibacterial agglutinins. This view is largely supported by the findings of Gibson (1930), but Boyd (1939-40) and Messer (1943) have drawn attention to the occurrence of agglutinins in considerable concentration in some "normal" rabbit sera. Indeed, with  $\alpha$  agglutinin, the natural antibody titres may be as high as those found in sera from immunised animals (Stamp and Stone, 1943-44; Francis and Buckland, 1945).

In the present work, certain paracolon bacilli isolated from desoxycholate-citrate-agar plate cultures during the examination of faeces from cases of enteritis or their contacts were found to have biochemical reactions superficially resembling those of certain specific pathogens and to be agglutinated to titre by a considerable number of batches of Oxford standard agglutinating sera prepared against different organisms. In preliminary absorption experiments with antisera to *Shigella sonnei* and *Shigella flexneri*, types Newcastle and Boyd 103, the paracolon bacilli failed to remove the specific agglutinins for the dysentery bacilli, and the homologous dysentery bacilli had no effect on the ability of the sera to agglutinate the paracolon strains. Subsequently the paracolon strains were found to be agglutinated to high titres by the sera of two healthy uninoculated rabbits. These rabbit sera also agglutinated lactose-fermenting and non-lactose-fermenting coliforms from human faeces or contaminated war wounds. The agglutinins in these sera were studied in absorption experiments and the results form the subject of this paper.

## Methods

*Strains.* Four strains of paracolon bacilli (PR, PW and PM from faeces, P 6 from a contaminated war wound) and 4 strains of *Bacterium coli* of Wilson *et al.* (1935), type I (CA and CB from faeces, C 5 and C 7 from wounds) were used for the main absorption experiments. Part of the work was repeated with three known  $\alpha$  strains, including the classical 1721, kindly provided by Dr Doris Stone.

*Bacterial suspensions.* The organisms grown on Lemco-agar slopes for 18 hours at 37° C. were washed off in saline (0.85 per cent. sodium chloride)

containing 0.5 per cent. formalin. Suspensions containing about 30,000 million organisms per ml. were used to absorb the sera and about 2000 million organisms per ml. for the agglutination tests.

*Rabbit sera.* The two healthy uninoculated rabbits were bled and their sera, designated 4 and 5, preserved by the addition of 0.01 per cent. acriflavine.

*Serum absorption.* To one part by volume of serum the absorbing suspension was added in three stages, 1, 3 and 5 parts by volume, at approximately 15-minute intervals, and absorption was done in a water-bath at 50° C. At the end of one hour the bacterial bodies were deposited by centrifuging and the serum, now diluted 1 in 10, was pipetted off. When multiple absorption was carried out the suspensions were first concentrated by centrifuging, so that the absorbed serum was not diluted more than 1 in 10.

*Agglutination experiments.* These were performed by the Dreyer method and were read after four hours' incubation in a water-bath at 50° C.

### *Experimental observations*

The selected strains were used singly to absorb separate samples of serum. After absorption, every sample was tested in turn with saline suspensions of each of the eight strains, which were found to fall into two distinct groups, I and II. Absorption with any one member of either group abolished or markedly reduced the ability of a serum to agglutinate any of the members of the same group, whereas members of the other group were agglutinated by the absorbed serum at titres as high as they were by the same serum unabsorbed (tables I and II). Moreover, in these experiments, the behaviour of

TABLE I

*Agglutinin-absorption tests with 8 selected coliforms and normal rabbit serum 4*

Agglutinable suspension	Serum 4 absorbed with							
	PR	PW	PM	CA	C 5	C 7	CB	P 6
PR	R	R	R	r	U	U	U	U
PW	R	R	R	r	U	U	U	U
PM	r	r	R	r	U	U	U	U
CA	r	r	R	U	U	U	r	U
C 5	U	U	U	U	R	R	R	r
C 7	U	U	U	U	R	R	R	R
CB	U	U	U	U	R	R	R	R
P 6	U	U	U	U	r	R	R	R

U = titre unchanged by absorption.

R = titre reduced by absorption to below 1 in 25.

r = titre reduced by absorption but not below 1 in 25.

individual members of the two groups was not uniform. Thus although a serum absorbed by any particular organism failed to agglutinate the absorbing strain at a titre of 1 in 25, it might still be capable of agglutinating other members of the same group at or above that

titre: A consideration of these results led to tentative deductions about the antigen and antibody complexes concerned.

TABLE II

*Agglutinin-absorption tests with 8 selected coliforms and normal rabbit serum 5*

Agglutinable suspension	Serum 5 absorbed with							
	PR	PW	PM	CA	C 5	C 7	CB	P 6
PR	R	R	R	r	U	U	U	U
PW	R	R	R	r	U	U	U	U
PM	R	R	R	r	U	U	U	U
CA	R	R	R	R	U	U	U	U
C 5	U	U	U	U	R	r	r	r
C 7	U	U	U	U	r	R	r	r
CB	U	U	U	U	r	r	R	R
P 6	U	U	U	U	r	r	R	R

For explanation of letters see footnote to table I.

In tables III-V the suggested antigenic formula for each strain is given in the second column of the table; the roman numerals represent common group antigens, the capital letters minor antigen factors. At the bottom of the columns from the third to the end, the corresponding group and minor antibodies in the untreated and absorbed sera are represented by arabic numerals and small letters. The results indicate either the dilution of serum giving standard agglutination or the absence of any agglutination at a dilution of 1 in 25.

TABLE III

*Agglutination and agglutinin-absorption tests with group-I coliforms and normal rabbit serum 4*

Agglutinable suspensions		Serum 4					
		unabsorbed	absorbed with				
Name	Antigens		PR	PW	PM	CA	PR+CA
PR	IA	2500	0	0	0	250	0
PW	IA	1000	0	0	0	250	...
PM	LAB	2500	50	50	0	125	0
CA	IB	1000	125	125	0	0	0
Serum antibodies		1 ab	b	b	none	a	none

Figures indicate serum dilution giving standard agglutination.  
0 = no agglutination at 1 in 25.

Table III shows results with serum 4 and the group I strains. These organisms fall into three sub-groups and possess one or other

or both of the minor antigens A and B in addition to the group antigen I. Thus with this serum, absorption with IA strains leaves agglutinins only for the strains possessing the B antigen; absorption with the IB strain leaves agglutinins only for the strains possessing the A antigen; and absorption with the IAB strain abolishes the power to agglutinate all members of group I, a similar result being obtained if the serum is absorbed simultaneously with IA and IB strains. Serum 5 contained no detectable b agglutinin and the a agglutinin appeared to be minimal (table IV). Similarly normal rabbit sera

TABLE IV

*Agglutination and agglutinin-absorption tests with group-I coliforms and normal rabbit serum 5*

Agglutinable suspensions		Serum 5				
		unabsorbed	absorbed with			
Name	Antigens		PR	PW	PM	CA
PR	IA	2500	0	0	0	25
PW	IA	1000	0	0	0	25
PM	IAB	2500	0	0	0	25
CA	IB	1000	0	0	0	0
Serum antibodies		1a	none	none	none	a

See footnotes to table III.

from Leeds and from the Institute of Animal Pathology, Streatley, which agglutinated IA and IAB strains, failed to agglutinate the IB strain.

TABLE V

*Agglutination and agglutinin-absorption tests with group-II coliforms and normal rabbit serum 5*

Agglutinable suspensions		Serum 5							
		unabsorbed	absorbed with						
Names	Antigens		C 5	C 7	CB	P 6	C 7 + P 6	C 5 + P 6	C 5 + C 7
C 5	II VX	2500	0	1000	500	1000	25+	0	0
C 7	II WY	500	250	0	50	125	0	25+	0
CB	II XY	1000	125	50	0	0	...	...	...
P 6	II XY	2500	1000	500	0	0	0	0	0
Serum antibodies		2vwxy	wy	vx	vw	vw	v	w	none

See footnotes to table III.

25+ indicates standard agglutination at a titre of 1 in 25 or higher.

Serum 5, however, clearly demonstrates the antigenic complexity of the group II coliforms. Table V shows that again there are three

sub-groups sharing a common group antigen II with one or other or both of two minor antigens X and Y; strains, however, which possess only one of these minor antigens have in addition a further antigen V or W. Thus absorption of serum 5 by any one group-II strain abolishes only the power to agglutinate that organism or a member of the same sub-group, the titres for the other members of the main group remaining in most cases quite high. If two strains are used simultaneously for absorption, only the combination of IIVX and IIVY strains removes all the relevant agglutinins; any other combination of two sub-groups leaves residual agglutinins for the third, which is agglutinated to a titre of at least 1 in 25. Detailed conclusions were not drawn from the results with serum 4 and the group-II strains (table VI) because of the relatively low titres of this

TABLE VI

*Agglutination and agglutinin-absorption tests with group-II coliforms and normal rabbit serum 4*

Agglutinable suspensions	Serum 4				
	unabsorbed	absorbed with			
		C 5	C 7	CB	P 6
C 5	1000	0	0	25	50
C 7	500	0	0	0	0
CB	500	0	0	0	0
P 6	2000	50	0	0	0

See footnotes to table III.

serum for strains C 7 and CB. It would appear, however, that serum 4 contained group-II and at least two minor agglutinins. Agglutinins for group-II strains were also found in the Leeds and Streatley rabbit sera.

The three  $\alpha$  strains supplied by Dr Doris Stone and one strain received from Dr A. I. Messer all fell into the IA sub-group. In this connection it is of some interest to note that this was also the antigenic structure of the only two strains which underwent spontaneous loss of agglutinability during this work.

Finally, serum 4 contained agglutinins for an old laboratory strain of *Shigella flexneri*. These "Flexner" agglutinins were not removed by absorption with either group-I or group-II strains, and absorption with the dysentery organism did not affect the agglutinins for the coliform or paracolon strains.

### Discussion

The sharing of antigenic factors between apparently unrelated organisms is being increasingly investigated (Bersohn and Lewin,



1945; Seligmann and Saphra, 1946), but some of the conclusions reached in other work of this kind do not appear to be entirely justified. Bridges and Taylor (1945-46) have already shown that "*B. wakefield*", which was believed to be serologically related to members of the Flexner group, did in fact possess the  $\alpha$  antigen and that cross reactions obtained by Berger (1945-46) were due to the presence of  $\alpha$  agglutinins in some of his agglutinating antisera. Similarly, the opinion of Weil, Binder and Slafkovsky (1946) that agglutinins for "*B. wakefield*" are produced in response to inoculation with *Shigella* sp. Sachs Q 454 is probably ill-founded. Sevitt (1945-46) reported that one of his paracolon strains was agglutinated "to full titre, with the Newcastle (Oxford) serum", but since both Bridges and Taylor (1945-46) and I had similar experiences with  $\alpha$  strains, it is not reasonable to conclude that such strains possess even minor components of dysentery bacilli.

Wheeler, Stuart and Ewing (1946) described five serological groups of coliforms which were agglutinated by sera prepared against *Shigella paradysenteriae* Boyd, type P 274. Since one of their antisera agglutinated "*B. wakefield*" at a titre of 1 in 5120 it may be that some of their cross agglutinations were due to  $\alpha$  agglutination. In a later communication Wheeler and Stuart (1946) reported that antisera to certain serological types of non-mannitol-fermenting members of the genus *Shigella* agglutinated coliforms which "possessed a Vi-like antigen present in Vi typhoid organisms and in certain coliform and paracolon cultures". This again suggests that  $\alpha$  agglutinins may have been a source of confusion.

The present work confirms that natural agglutinins may be present in considerable concentrations in the sera of uninoculated rabbits. Moreover, since evidence is produced for the presence of agglutinins here designated 1, 2, a, b, "Flexner" and probably two others in serum 4 and 1, 2, a, v, w, x and y in serum 5 it appears that such natural agglutinins may be of considerable complexity. Although Oxford standard diagnostic sera are now absorbed with  $\alpha$  strains to minimise the risk of errors in identification (Bensted, personal communication), it has now been shown that the classical  $\alpha$  strain 1721 belongs to the present IA sub-group and will not exhaust all naturally-occurring agglutinins for the IB and IAB members of the same main group. The demonstration in normal rabbit sera of a second complex of agglutinins capable of reacting with other Gram-negative bacilli serologically unrelated to the  $\alpha$ -containing group, reinforces the view that absorption with a limited number of agglutinable strains cannot be relied upon to eliminate all such sources of error.

If rabbit antisera are used for analytical work on the sharing of antigens by different bacteria, the results should be controlled with sera obtained from the animals before inoculation. This precaution was recently observed by Ferguson and Wheeler (1946).

## Summary

In absorption experiments with sera from uninoculated rabbits and coliform and paracolon bacilli agglutinated by these sera, the organisms investigated fell into two distinct serological groups, each containing three sub-groups.

Three  $\alpha$  strains received from Dr Doris Stone belonged to the same sub-group.

The "colon-paracolon" agglutinins were not those responsible for the agglutination of an old laboratory strain of *Shigella flexneri* by one normal serum.

At least seven distinct agglutinins were recognised in each of the two normal rabbit sera investigated.

I wish to thank Dr Doris Stone and Dr A. I. Messer for  $\alpha$  strains and Miss C. L. Clark and Dr K. Zinnemann for normal rabbit sera from Streatley and Leeds.

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# THE QUANTITATIVE DESCRIPTION OF THE FRAGILITY OF THE ERYTHROCYTE AND ITS APPLICATION TO THE STUDY OF ACHOLURIC JAUNDICE

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SEVERAL quantitative studies of the lysis of erythrocytes by hypotonic solutions have been reported, notably by Whitby and Hynes (1935), Vaughan (1937), Creed (1938), Dacie and Vaughan (1938), and Colli (1945). Two fundamentally different methods have been used: that of Simmel (1923), in which the lysis is determined by counting the number of erythrocytes which survive treatment with a given solution and comparing the result with a similar dilution in Hayem's solution; and those of Momigliano-Levi (1935) and Creed (1938), in which the hæmoglobin liberated is measured. Since the volume and concentration of hæmoglobin in the different cells varies, these two methods measure "fragility" in terms of two incommensurates and might therefore yield quite different results on the same blood. Further, though it is not particularly difficult to compare the concentration of two solutions with an accuracy of  $\pm 3$  per cent., Lyon and Thoma (1881) have pointed out that the estimation of erythrocytes in a suspension is much less simple, for, apart from errors of calibration, the statistical error is such that to reach an accuracy of  $\pm 3$  per cent. some 5000 cells must be counted at each concentration tested; to enumerate some 60,000 cells for each complete determination is quite impracticable. It is evident that for precise work a hæmoglobinometric method is essential, either that of Creed, or one which uses sufficient fluid for photometric or absorptionometric determination of the liberated hæmoglobin, such as that of Hunter (1940).

All observers agree that if the lysing concentration of salt be plotted in rectangular co-ordinates against the percentage of hæmoglobin liberated, or against the percentage of cells lysed, then the experimental points lie on or close to a smooth sigmoid curve, from which it is easy to read off the concentration of salt which causes 50 per cent. lysis—the M.C.F. or mean corpuscular fragility. Attempts to describe the range over which hæmolysis occurred—the "span"

of fragility—were unsuccessful, so that Dacie and Vaughan specify the range of salt concentration which covers 16 to 84 per cent. lysis—essentially an arbitrary range, while Gallo (1945) developed an empirical equation to fit Colli's figures.

In 1940, Hunter claimed that if the results were plotted on arithmetic probability paper the curve became a straight line, and deduced that the fragility curve, as experimentally determined, is the first integral of the probability distribution of erythrocyte fragility with respect to salt concentration. If this be so, then, if the intervals of salt concentration were reduced so as to be very small and if the difference between percentage lysis at one concentration and that at the next lower concentration were plotted against salt concentration, the resulting curve—the *differential curve of fragility*—would approximate closely to the normal (Gaussian) curve of errors, characterised completely by two parameters, the mean and the standard deviation. Such distribution curves are usual in measurements which are summations of the effect of large numbers of similar but distinct events, and the use of arithmetic probability paper in describing them has a practical value, since with a straight line one may interpolate, or even extrapolate, with considerable accuracy; in the normal distribution of errors a range of  $\pm$  twice the standard deviation about the mean includes 95 per cent. of the area under the curve; so that if the concentrations of salt which produce 2.5 per cent. and 97.5 per cent. lysis are read off, the difference between them is equal to four times the standard deviation. The fragility of erythrocytes should be described completely by determining the M.C.F. and its standard deviation.

Recently, Parpart *et al.* (1947) reported a more detailed study of fragility, confirming Hunter's observations, and showing that pH and temperature are important variables; their method, using buffered salt solutions, appears likely to become the standard method. In the present study unbuffered salt solutions, initially at pH 6.4, were used to study the fragility of normal erythrocytes and erythrocytes from patients with acholuric jaundice (familial hæmolytic anaemia) and other anaemias.

#### *Method (modified from Hunter)*

Venous blood containing 2 mg./ml. of Heller and Paul's (1933-34) oxalate mixture is aerated by rotation for 4-6 mins. in a 6 in.  $\times$  1½ in. tube. Into each of a series of tubes containing 10 ml. of the different concentrations of sodium chloride is measured 0.1 ml. of blood, the tubes stoppered, mixed by inversion, and after 30-45 mins., centrifuged at 2000-3000 *r.p.m.* for about 10 mins. The supernatants are compared in a photo-electric absorptiometer, using an Ilford spectrum yellow (no. 606) filter, and a dilution of 0.1 ml. of blood in 10 ml. of 0.1 per cent. sodium carbonate is used as a standard. It had been ascertained that with this filter, which isolates light absorbed by the  $\alpha$ -band of oxyhæmoglobin, the Lambert-Beer law is followed. The salt solutions, of interval of 0.02 per cent., were prepared from a 1.0 per cent. solution checked by titration with silver nitrate, using dichlorfluorescein as indicator.

## Results

This is a most troublesome analysis, as hæmolysis sometimes occurs in a single tube for no apparent reason. It was found necessary to measure out the salt solutions and let them stand overnight before satisfactory duplicates could be obtained. Measurements of slight hæmolysis are inaccurate owing to the small galvanometer deflection, while when more than 95 per cent. hæmoglobin is liberated, measurements again become inaccurate owing to the peculiarities of the scale

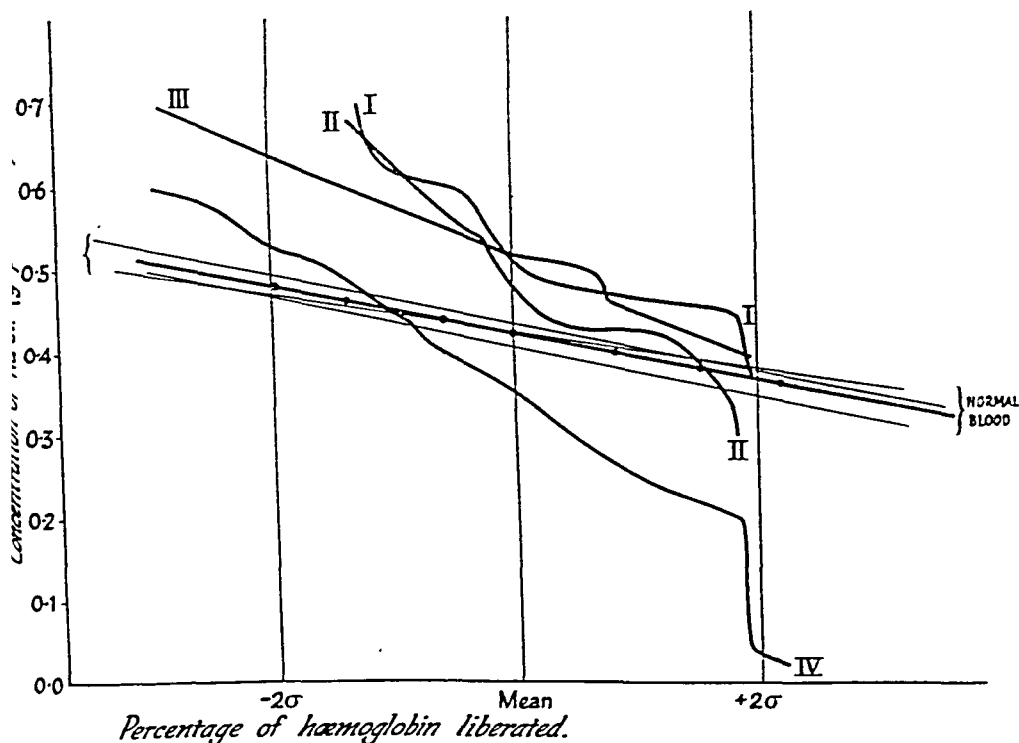


FIG. 1.—Fragility curves plotted on arithmetic probability paper. Four normal subjects not individually labelled. I and II, classical acholuric jaundice before splenectomy; III, the same 14 days after splenectomy; IV, largest range of fragility encountered. Mean = 50 per cent.,  $-2\sigma = 2.5$  per cent.,  $+2\sigma = 97.5$  per cent. Hb. liberated.

of extinction; reliable results are obtained only between 5 and 95 per cent. hæmolysis. Bearing in mind these limitations (which are present in some degree in all methods) the results for normal blood do not disagree with Hunter's statement. The experimental points fall on, or close to, straight lines (fig. 1); the M.C.F. and standard deviation obtained from 10 normal subjects are given in table I, together with estimates of the range which should include 95 per cent. of all normal subjects, calculated by the method of Maskell (1930, quoted by Fisher, 1942).

These results agree satisfactorily with those of Whitby and Hynes and of Colli, but the values of M.C.F. are higher than those of Dacie

and Vaughan. Since these authors used a dilution of 1 part of blood in 25 parts of saline, this difference is probably due to a difference in final salt concentration.

TABLE I

*Mean corpuscular fragility and standard deviation of fragility for ten normal subjects*

M.C.F. (percentage NaCl)	Standard deviation (percentage NaCl)
0.438	0.03050
0.435	0.02750
0.435	0.02700
0.430	0.02900
0.429	0.03250
0.425	0.02975
0.424	0.02300
0.422	0.02825
0.412	0.03000
0.408	0.03050
Mean 0.4258	0.02880
Maximum 0.4484	0.03468
Minimum 0.4032	0.02292

A variety of anæmic patients were investigated by means of this technique. In the majority the M.C.F. was within normal limits, though the standard deviation of fragility was increased, but in only one patient did it exceed 0.046 per cent. (curve IV, fig. 1). Examination of a family of patients suffering from acholuric jaundice (fig. 2),

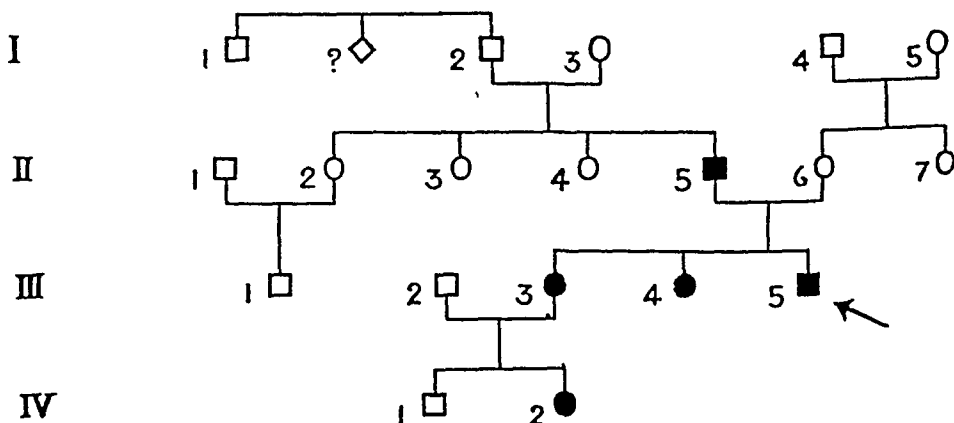


FIG. 2.—Family tree. Symbols of Eugenics Society: males, squares; females, circles; sibs whose sex is unknown or not significant, diamonds; solid squares or circles, abnormality present. Arrow indicates proband. Successive generations numbered in roman numerals.

however, gave rather surprising results, which may be of some diagnostic importance.

## Case summaries (see fig. 2)

Case. III 5. The propositus, III 5, aged 15 years, was admitted to Hill End Hospital on 1st February 1946 with a left-sided lobar pneumonia and pleural effusion, which responded satisfactorily to treatment. He gave a history of attacks of jaundice lasting from 2 days to 2 weeks, during which he felt a distaste for fatty foods and passed dark urine; his first attack had occurred in January 1944, the most recent on 23rd December 1945. His spleen and liver were palpable 2 in. below the costal margin and the significant laboratory reports showed constant slight urobilinogenuria, constant reticulocytosis fluctuating between 3.2 and 11 per cent.; erythrocyte fragility increased on two occasions—M.C.F. 0.483 and 0.467 per cent., standard deviation 0.0325 and 0.025 per cent. respectively; Coomb's test for autohaemolysins negative. Splenectomy was performed by Mr J. P. Hosford on 30th April: recovery was uneventful.

The other members of the family were seen at least once and the results of the laboratory examinations are given in table II.

TABLE II

*Hæmatological data of members of the family studied*

Subject	Reticulo- cytes (per cent.)	Fragility		Halo- meter ( $\mu$ )	Price-Jones curve					Cell thickness ‡		
		M.C.F.	S.D.		Mean ( $\mu$ )	S.D.	Micro- cytosis (per cent.)	Macro- cytosis (per cent.)	$g_1$ †	MCV	MCT	MCT/ MCD
II 1	0.1	0.430	0.029	7.3	...	...	...	...	...	...	...	...
II 2	<0.1	0.435	0.0275	7.2	7.215	0.4392	0	0.2	3.1	...	...	...
II 3	0.6	0.458	0.0312	7.15	7.125	0.4642	0	0	0.48	...	...	...
II 4	0.5	0.449	0.028	7.2	7.335	0.4579	0	0	0.052	...	...	...
II 6	0.1	0.424	0.023	7.0	7.1175	0.4268	0	0	4.1	100	2.52	2.82
III 1	0.3	0.435	0.027	7.1	7.0995	0.4266	0	0	1.67	...	...	...
IV 1	1.3	...	...	7.3	7.0475	0.5237	1	0	16.7	...	...	...
II 5	3.5	0.480	0.0235	6.8	6.910	0.641	5.4	0.6	2.65	92.7	2.48	2.78
III 3	6.0	0.479	0.046	6.55	6.4825	0.4965	14.8	0	0.447	90.4	2.71	2.38
III 4	6.7	0.480	0.0325	6.15	6.5545	0.565	17.8	0	1.3	102.7	3.15	2.16
III 5*	0.2	0.486	0.025	6.5	6.6825	0.4819	7.4	0	1.73	115.5	3.29	2.03
IV 2	3.0	...	...	...	...	...	...	...	...	...	...	...

\* Five months after splenectomy.

† In terms of its standard deviation—a measure of asymmetry.

‡ Measured with apparatus not standardised by N.P.L.

Case II 5, aged 49, had always enjoyed perfect health; he would admit no symptoms suggestive of anæmia or of gall-bladder disease. His spleen was not palpable clinically and radiological examination showed its size to be within the limits of normal.

Case III 3, aged 24, had always been healthy and her two confinements had been normal. Her spleen was not palpable nor was it enlarged radiologically.



Case III 4, aged 23, had always been healthy. Her spleen was palpable on full inspiration and grossly enlarged radiologically. In March 1947 she developed a slight and evanescent jaundice.

Case IV 2, aged 2, was healthy and had never been jaundiced. Her spleen was not palpable nor enlarged radiologically, but full hæmatological examination was not performed because she objected vigorously.

The subjects I 5, II 1, 2, 3, 4, 6, III 1 and IV 1 were all examined and found normal.

### *Discussion*

The fragility curves of normal subjects and of affected members of the family T (fig. 2), yield, when plotted in rectangular co-ordinates, smooth sigmoid curves; or, on arithmetic probability paper, straight lines. Comparable curves from patients with classical acholuric jaundice vary considerably in form (fig. 1, I and II before, III after splenectomy), but usually show only a small proportion of hæmoglobin liberated at high concentrations of NaCl (*cf.* Dacie and Vaughan), while most of the hæmolysis occurs quite close to the normal range. Such curves may be closely imitated if one regards them as the sum of two or three fragility curves, each curve with its own M.C.F. and standard deviation. This suggests that in classical acholuric jaundice two or three groups of cells, differing in M.C.F. and its standard deviation, co-exist and together make up the red cell population studied.

It is doubtful whether transition between the classical form of acholuric jaundice with a bi- or trimodal fragility curve and the unimodal form exemplified by family T can occur. In the classical form, increase in fragility appears constant except during intense hæmolytic crises. If transition between the two forms did occur, the chance of finding both in this family would be appreciable. Indeed, if the two phases were of equal duration the chances would be 15 to 1 against finding the results reported here. Further, one other unrelated patient with a unimodal curve has been seen, and over a period of some years his fragility has been qualitatively only just recognisably increased.

An attempt was made, on the lines laid down by Race (1941-42), to demonstrate linkage with OAB, Rh blood groups, salivary secretion of AB group-specific substance, ability to taste phenylthiocarbamide 1:20,000, presence of brown pigment in the iris, presence of hair on the middle phalanges, hyperextensibility of the thumb and attachment of the ear lobes. The blood and saliva were examined by Dr Sylvia Lawler of the Medical Research Council Blood Group Research Unit, to whom I am indebted for permission to quote her results. The data obtained are given in table III. No individual with hyperextensible thumbs, and only one with attached ear lobes, was observed: all were right-handed. All affected individuals were secretors of blood-group substance (except IV 2, who was not tested), but this only

indicates that future studies of this condition should include this test. The results are too few to justify any comment.

TABLE III

*Tests for linkage with genetically determined factors*

Subject	OAB	Rh	Saliva	PTC tasting	Eye pigment	Hair on phalanges
II 1	A <sub>2</sub>	R <sub>1</sub> R <sub>1</sub>	ss	tt	+	+
2	A <sub>1</sub>	R <sub>2</sub> r	ss	T	—	+
3	0	rr	...	T	—	—
4	0	R <sub>2</sub> r	...	tt	+	+
5	A <sub>1</sub>	rr	S	tt	—	—
6	A <sub>1</sub>	R <sub>1</sub> r	ss	T	+	—
III 1	A <sub>1</sub>	R <sub>1</sub> r	ss	tt	+	+
3	A <sub>1</sub>	R <sub>1</sub> r	S	T	—	—
4	A <sub>1</sub>	R <sub>1</sub> r	S	T	+	—
5	A <sub>1</sub>	R <sub>1</sub> r	S	T	—	—
IV 1	A <sub>1</sub>	R <sub>2</sub> R <sub>1</sub>	S	tt	—	—
2	0	R <sub>1</sub> R <sub>1</sub>	...	T	—	—

### Conclusion

One may submit, as a provisional hypothesis, that familial acholuric jaundice with its unimodal fragility curve appears to be genetically distinct from the more usual form of the disease, though it is inherited in the same way as an autosomal character difference with marked single dose expression. Clinically it differs from the classical form in that splenomegaly is inconstant, and the manifestations of the disease are extremely mild.

### Summary

1. In normal subjects the osmotic fragility is normally distributed about a mean, and can be described completely by stating the mean corpuscular fragility and its standard deviation.
2. In classical acholuric jaundice the fragility is not normally distributed and there appears to be more than one population of red cells present.
3. A family is described, several of whose members suffer from acholuric jaundice, but who present a unimodal fragility curve.
4. It is suggested that this family is affected by a form of acholuric jaundice genetically distinct from the classical form of the disease.

I am indebted to Dr E. R. Cullinan for permission to publish this account of his patient, and to Dr George Simon for the interpretation of the radiographs.

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## SHORT ARTICLES

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### ADDISON'S DISEASE DUE TO CONGENITAL HYPOPLASIA OF THE ADRENALS IN AN INFANT AGED 33 DAYS

H. ŠIKL

*Prague*

(PLATES LVI AND LVII)

Aplasia or high-grade hypoplasia of the adrenals is known to occur in anencephalic monsters and other forms of severe malformation of the central nervous system. As these developmental defects are incompatible with post-natal life, Addison's disease fails to make its appearance. During foetal life the maternal adrenals are obviously able to substitute for the deficient adrenal function of the foetus.

There is, on the other hand, in the older literature (Crosby, 1930) a number of cases of Addison's disease in adults in which the adrenals could not be found at the post-mortem. Such cases are generally considered as congenital aplasia of the adrenals. This view, however, is open to criticism, since, in the light of present knowledge of adrenal function, it is scarcely admissible that an individual with adrenal aplasia could reach adult age. It is far more probable that in these cases the condition of the adrenals was acquired during post-foetal life, and was of the nature of inflammatory or non-inflammatory atrophy. Some degree of congenital hypoplasia, however, may be assumed as a basis for later involvement of the adrenals in pathological processes of various kinds, including even tuberculosis, which is generally admitted to develop on the basis of an inborn inadequacy of the adrenal glands. This may apply particularly to cases of Addison's disease in younger children, of which Chemin (1910) was able to collect 55 examples. The youngest cases so far known seem to be those of Belyaev (7 days) and Cannata (18 months) (both cited from Klein and Kux, 1932).

Newer observations of this kind are those by Klein and Kux (12-year-old female with tuberculous destruction of the adrenals), Casaubon and Cossoy (1940) (12-year-old female), as well as Rosin and Friedman (1943) (7-year-old male; no autopsy). Wakefield and Smith (1927) mention a family in which, in addition to a case of Addison's disease in an adult, seven males in four generations had bronzing of the skin from birth.

#### *Case report*

My own case is that of a male infant who died at the age of 33 days. Unfortunately the patient could not be given adequate clinical study as he was moribund on admission and died the same day. According to the mother's statement, subsequently elicited, the child was born at full term and weighed 3700 g. It appeared healthy at first but did not take the breast well, and it gained very little weight in the first two weeks. At three weeks a greyish-brown discolouration of the skin became apparent and grew deeper until death. Asthenia was not conspicuous. Later the baby developed diarrhoea and the course was steadily downhill.

On admission the child appeared decidedly ill-nourished, weighing only 3000 g. There was marked discolouration and much scaling of the skin. The heart's action was slow and the sounds were weak.

The post-mortem (1806/45), performed 20 hours after death, disclosed rather severe enteritis and confluent bronchopneumonia. The skin showed conspicuous brownish pigmentation which was partly masked by scaling. There were some pigmented spots in the oral mucosa. Sections of the skin stained by Masson's method for melanin (fig. 1) clearly showed the accumulation of pigment in the epidermis with none in the corium, as is characteristic of Addison's disease.

The discolouration of the skin naturally directed attention to the adrenals, which were found to be very hypoplastic, measuring only  $10 \times 8$  and  $12 \times 8$  mm. respectively, and little more than 1 mm. in thickness (fig. 2). In order that the specimen might be preserved for the museum the adrenals were not weighed and only a thin slice was taken from each for microscopic examination.

Fig. 3 shows the whole of one adrenal in transverse section, with a normal organ of the same age for comparison. One may estimate the volume of the hypoplastic organ as being only about one-hundredth of the normal. With higher power (fig. 4) the architecture of the adrenals appeared rather irregular. Cortex and medulla could not be distinguished, and there were areas of highly vascular loose fibrous tissue. The trabecular arrangement was indicated only in places and many of the individual cells showed definite hypertrophy. No signs of inflammation or of old blood-pigment could be detected.

At first sight one had the impression that the whole of the tissue might be composed of medullary cells. To decide this we made use of the stain suggested by Bodian for nerve axon fibres. As was shown by my colleague Dr Fingerland (1940), this method gives very selective staining of the medullary cells, the plasma of which appears filled with purplish-black granules, while the nuclei remain unstained. This contrasts with the cortical cells, in which the nucleus is clearly stained but there are no granules in the plasma. This stain, when applied to our case (fig. 5), showed that the tissue was mainly composed of cortical cells but that there were scattered single medullary elements, particularly in the vascular areas.

The hypophysis appeared microscopically normal, and the degree of differentiation of the chromophilic cells was approximately the same as that of a normal child of this age. There were no obvious changes in the testes.

### Summary

We believe that this is a case of true congenital hypoplasia of the adrenals and not the result of hemorrhagic destruction of these glands at birth. Although adrenal insufficiency may not have been the immediate cause of death, we consider that the bronzing of the skin and pigmentation of the oral mucosa, together with the poor state of nutrition and weak heart action, warrant the diagnosis of Addison's disease.

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 E. E.

## ADRENAL HYPOPLASIA



FIG. 1.—Skin from thorax stained by Masson's method for melanin.  $\times 110$ .



FIG. 2.—Gross specimen, showing hypoplasia of adrenals. From each gland a thin slice has been taken for microscopic examination.  $\times 1.4$ .



FIG. 3.—Transverse section of hypoplastic left adrenal (inset) compared with a similar section from the normal organ of an infant of the same age. Hæmatoxylin and eosin.  $\times 6$ .



## ADRENAL HYPOPLASIA

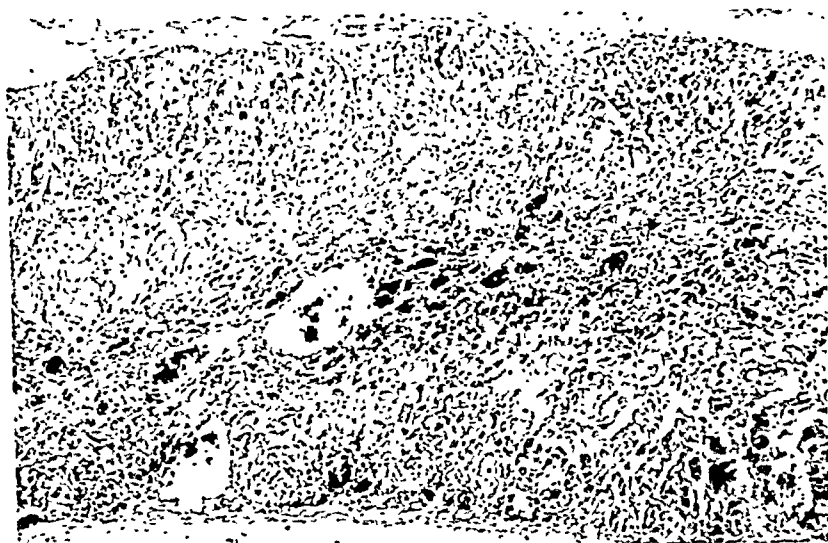


FIG. 4.—Section of hypoplastic adrenal, showing the irregular arrangement of the parenchyma cells, which are partly hypertrophied. There are areas in the centre of the gland of highly vascular tissue in which no medulla can be identified. Hæmatoxylin and eosin.  $\times 20$ .

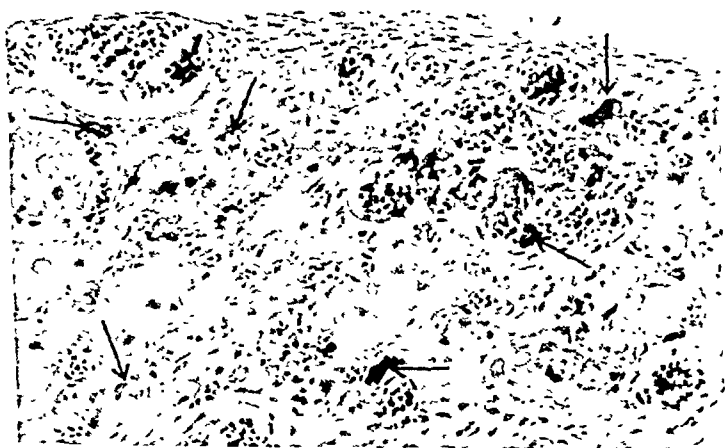


FIG. 5.—Section of the vascular tissue shown in fig. 4. Scattered isolated medullary cells are indicated by arrows. Cortical cells (to left) are very faintly stained. Bodian's stain.  $\times 200$ .





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THE HISTOLOGICAL DEMONSTRATION OF GLYCOGEN IN  
NECROPSY MATERIAL

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Considerable confusion surrounds the question of the rapidity with which glycogen disappears from the tissues after death and the behaviour of this substance in different fixatives, particularly formaldehyde. The prevalent text-book conception is well exemplified by Boyd (1944, p. 334). "Glycogen in the normal liver is notoriously difficult to demonstrate, unless it is examined for immediately after death and the tissue is fixed in absolute alcohol, for the glycogen is rapidly converted into glucose by this glycogenolytic ferment and as it is soluble in water it is dissolved out by a watery fixative such as formalin". Yet Külz (1880-81) was able to estimate glycogen in considerable quantity in the unfixed liver of various animals after it had been kept at room temperature for as long as eight days after death. It appears from this early observation and from later work (Garnier and Lambert, 1897; Bobbitt and Deuel, 1940; Swensson, 1945) that, although glycogen is lost from the tissues after death, the disappearance is far slower than is generally supposed. Romeis (1932) states that the setting free of glycogen after death is greatly hastened by the action of water; all contact with water or physiological saline is to be avoided, but he adds that Arndt recommends 4 per cent. formaldehyde saturated with grape-sugar as a fixative. Popper and Wozasek (1930-31), using 5 per cent. formaldehyde as a fixative in a few cases, obtained satisfactory demonstration of glycogen with Best's carmine stain, though they thought there was some loss in comparison with alcohol-fixed tissue. Brian *et al.* (1937) succeeded in demonstrating glycogen in slices of heart muscle, liver and kidney kept for two months in dilute formalin (U.S.P. 1:10). Bouin's fixative has been recommended for this purpose (Lison, 1936) and has been successfully used in this Institute for several years.

The following investigations were made in order to find out to what extent the paraffin blocks of formol-fixed material, collected from necropsies in previous years, could be used in an investigation of the glycogen content of the liver.

The principal factors requiring control are :—

1. Length of time between death and fixation of tissue. Many of our necropsies are performed 18-48 hours after death, the body being preserved meanwhile in the cold chamber.
2. Exposure of tissue to air at room temperature. At necropsy the organs may be so exposed for 1-2 hours before portions are fixed.
3. Length of time in formaldehyde. Though it is undesirable that blocks should be kept for more than a few days in this fixative, there have been periods when, for various reasons, the time has been extended to weeks or months.
4. Washing in water overnight before taking the blocks through graded alcohols. This step has been omitted in this Institute since 1930.

*Technique*

Two rabbits, each of approximately 2 kg., were fed on half-a-pound of carrots in addition to their usual food for three successive days. After the last feed the rabbit was anaesthetised with chloroform until muscular relaxation was

complete, the thorax opened and the heart excised. The liver was at once removed and pieces of the organ treated as follows:—

1. Immediate fixation (*i.e.* within 10 minutes) in absolute alcohol and Bouin's fluid respectively for standard controls.

2. Fixation similarly in 4 per cent. formaldehyde for 24 and 48 hours, and 4, 8, 16 and 38 days.

3. Exposure to air on the bench at room temperature for two hours and then fixation, (*a*) in formaldehyde, (*b*) in Bouin's fluid.

4. The remainder of the liver was meanwhile placed in a jar with a ground-glass stopper and put in the refrigerator at a temperature of about 3° C. After 18 and 48 hours respectively pieces were fixed in (*a*) formaldehyde and (*b*) Bouin's fluid.

After 24 hours in each fluid the tissue was transferred to 90 per cent. alcohol, thereafter to three changes of absolute alcohol, one hour in each, left in chloroform overnight and embedded in paraffin wax. Also, after 4 days in formalin, pieces were placed in water for 12 hours before being taken through the graded alcohols and embedded.

Sections of an average thickness of 6 or 7  $\mu$  were floated on 70 per cent. alcohol at 55-60° C., mounted and dried, and after removal of the paraffin were covered with a film of 1 per cent. celloidin. They were then stained with hæmatoxylin and Best's carmine (see Carleton and Leach, 1938, pp. 106 and 171). Two of the sections, fixed in Bouin's fluid and formalin respectively, were covered with celloidin and treated with saliva for 15 minutes before staining.

### Results

The saliva-treated sections were devoid of glycogen. All the others contained large amounts of glycogen in the cytoplasm of the liver parenchyma. The glycogen particles were smaller in the alcohol-fixed tissue than in the tissue fixed by other methods. With these exceptions there were only slight qualitative differences in the histological appearance of the remainder of the series.

An attempt was made to grade them in respect of the amounts of glycogen present, with the following results.

TABLE

*Effect of various methods of fixation on liver glycogen*

Method of fixation and other treatment	Rabbit A *	Rabbit B
Bouin's fluid 24 hours . . . . .	+++++	+++++
Alcohol 24 hours . . . . .	+++++	+++++
Formalin 24 and 48 hours . . . . .	+++++	+++++
" 4, 8, 16 and 38 days . . . . .	+++++	+++++
2 hours in air, Bouin or formalin 24 hours . . . . .	+++++	+++++
Refrigerator 18 hours, Bouin 24 hours . . . . .	+++++	+++++
" 18 " formalin 24 hours . . . . .	+++++	+++++
" 48 " Bouin 24 hours . . . . .	+++++	+++++
" 48 " formalin 24 hours . . . . .	+++++	+++++
Water 12 hours, after formalin 2 and 4 days . . . . .	+++++	+++++
Treated with saliva, Bouin 24 hours . . . . .	—	—
" " formalin 24 hours . . . . .	—	—

\* Rabbit A was killed during a spell of hot summer weather.

### Discussion

The allocation of only 4+ to the alcohol controls is perhaps due to the more finely particulate character of the glycogen in these sections, together with shrinkage of the cellular elements. Rabbit A was killed during a spell of hot

summer weather, which may explain the apparent greater loss on exposure of the tissue to air than in rabbit B. The results in rabbit B were in fact remarkably uniform. Taken as a whole there can be no doubt that formaldehyde is as efficient a fixative for glycogen as Bouin's fluid or absolute alcohol. In it there is only slight deterioration after as long as 38 days, while treatment of the tissue in running water appears to entail no loss of glycogen. No appreciable loss followed storage in the ice chest up to 48 hours.

The implications of these observations are both practical and theoretical. On the practical side it is clear that any formalin-fixed tissues embedded in paraffin can be stained for glycogen provided that the sections are cut and mounted in the appropriate way. Theoretically it appears desirable to modify the current idea that the glycogen stored in von Gierke's disease is in some way different from ordinary tissue glycogen, or, alternatively, that it exists under different conditions of storage. For this idea is largely based on the persistence of the glycogen for considerable periods in formaldehyde, *e.g.* 3 weeks (Ellis and Payne, 1936). There appears to be nothing remarkable in this in the light of the above observations.

I should like to thank Professor Dorothy Russell for her invaluable encouragement and criticism, and Mr Victor Trenwith for technical assistance.

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576 . 851 . 42 : 615 . 778

A SIMPLE METHOD FOR TESTING DYE SENSITIVITY OF  
BRUCELLA SPECIES

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(PLATE LVIII)

The ability of *Br. melitensis*, *Br. abortus* and *Br. suis* to grow in the presence of certain aniline dyes forms one of the most important criteria for distinguishing these species. The dye-sensitivity test, first described by Huddleson and Abell (1928), was further developed by Huddleson (1929, 1931), and, although subjected to some criticism by certain workers, has been favourably reported upon by the majority of those who have used it (Topley and Wilson, 1946).

The technique usually employed for determining the bacteriostatic action of the dyes is that recommended by Huddleson. Dyes obtained from the National Aniline Chemical Company of New York, or standardised against dyes obtained from that source, should be used. Stock solutions in 50 per cent. alcohol of thionin, basic fuchsin and methyl violet (1.0 per cent.) and of pyronin (0.5 per cent.) are prepared. For use, appropriate amounts of these solutions are added to liver-infusion agar, pH 6.6, to make dye plates containing 1 in 30,000 and 1 in 60,000 of thionin, 1 in 25,000 and 1 in 50,000 of basic fuchsin, 1 in 50,000 and 1 in 100,000 of methyl violet and 1 in 100,000 and 1 in 200,000 of pyronin. Heavy inocula of the organisms to be tested are spread over areas 1 cm. in diameter on these plates and on a control plate without dye. The amount of growth is observed after 3 days' incubation.

For this technique, nine plates are required if the four dyes are used. Further, the preparation of the plates is troublesome, since the agar and the dye solutions have to be kept near boiling point before mixing, the glassware has to be handled hot and the plates have to be rocked gently from time to time during the setting process to prevent precipitation of the dye.

*Simplified method*

The method now described was devised as a rapid and economical method of testing strains without elaborate preparation. The filter-paper strips which are used can be prepared and stored until required.

Strips of filter paper (Postlip 633) measuring  $6 \times 0.5$  cm. are placed in Petri dishes and sterilised in the autoclave. Each strip is then picked up with sterile forceps and one end dipped in an aqueous solution of the dye, which rapidly saturates it. The papers are replaced in the dish and dried in the 37° C. incubator overnight. The dye strips, which can be kept in the Petri dishes or in screw-capped bottles, remain effective for an indefinite period. The concentrations of the dyes which have been found satisfactory for impregnation of the paper are as follows:—thionin 1 in 800, basic fuchsin 1 in 200, methyl violet 1 in 400 and pyronin 1 in 800.

For use, strips impregnated with each of the dyes are laid in parallel, equally spaced, on the surface of a plate of liver agar, and a tube of the same medium (12 ml.), melted and cooled to 50° C., is poured on top. When the agar has set the plate is dried in the 37° C. incubator.

Milky suspensions of the brucella strains to be tested, including known strains of *Br. melitensis*, *Br. abortus* and *Br. suis*, are prepared by adding about

## DYE SENSITIVITY OF BRUCELLA

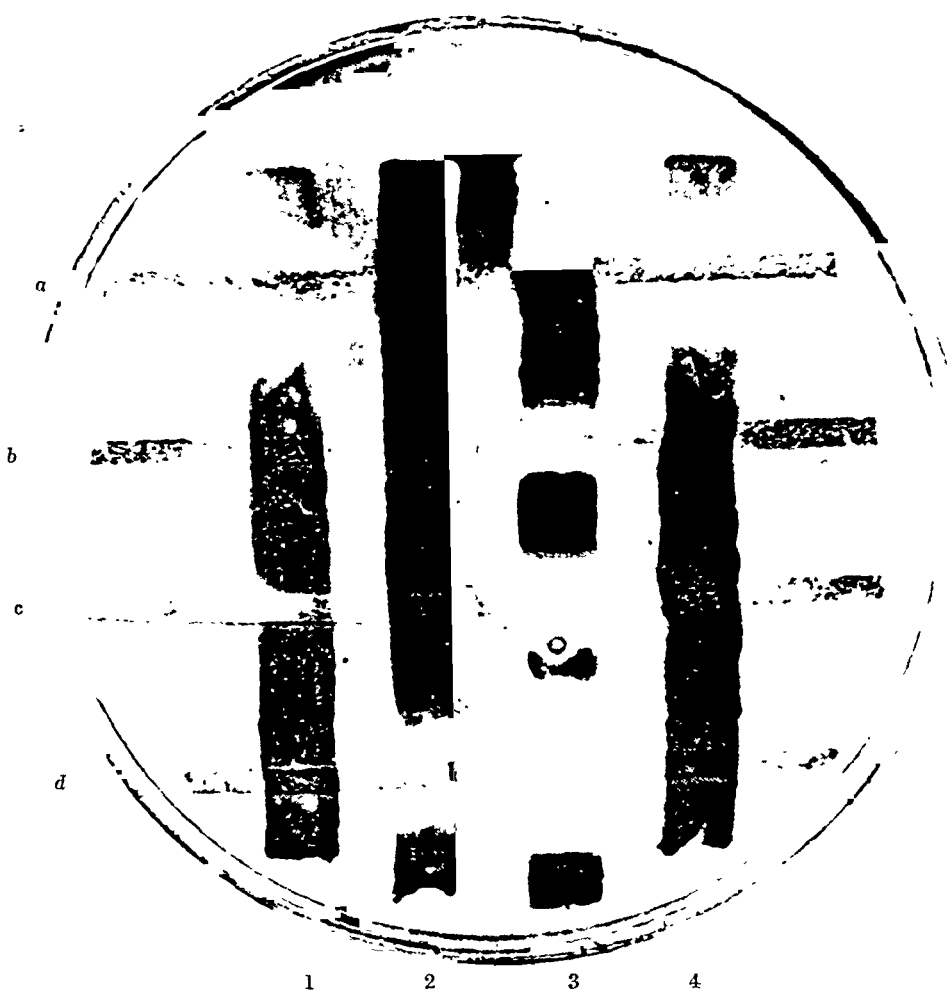


FIG.—Bacteriostatic action of dyes on *Br. abortus* (two strains), *Br. melitensis* (a pyronin-sensitive strain) and *Br. suis*. A 6-inch (15 cm.) Petri dish has been used.

## ORGANISMS

1. *Br. abortus*
2. *Br. melitensis*
3. *Br. suis*
4. *Br. abortus*

## DYES

- a. Thionin
- b. Basic fuchsin
- c. Methyl violet
- d. Pyronin



one ml. of broth to liver-agar slope cultures grown for 2 days in screw-capped bottles and emulsifying some of the growth with a loop. The dye plate, previously marked into sections with a grease pencil, is tilted slightly and inoculated with the brucella suspensions in strips at right angles to the filter papers by means of a flat loop spreader 8 mm. in width. When dry the plates are incubated at 37° C. in 10 per cent. CO<sub>2</sub> and inspected after 2 and 3 days. The results are usually distinct after 2 days' incubation. Up to six strains may be tested on one 4-inch (10-cm.) plate.

### Results

A typical result is shown in the figure. Photography of the plates is difficult owing to the range of colours of the four dyes. It will be noted that organisms not susceptible to the dyes grow freely up to and across the strip, and that sensitivity is indicated by inhibition of growth extending for distances up to 10 mm. from the edge of the strip.

Numerous recently isolated strains of *Br. abortus* and *Br. melitensis*, and a number of stock strains of these species and of *Br. suis*, have been examined in parallel by this method and the usual Huddleson technique. The two methods have yielded similar information in regard to dye sensitivity. The use of control strains of known behaviour is of course essential.

The majority of strains of *Br. abortus* are inhibited by thionin but grow in the presence of the other dyes; certain strains from Southern Rhodesia have greater resistance to thionin and a small number of strains are unduly susceptible to all dyes. Most strains of *Br. melitensis* grow on all the dyes but some sensitivity to pyronin and occasionally to thionin and methyl violet is not infrequent. Strains of *Br. melitensis* which are sensitive to thionin are also sensitive to methyl violet and may thus be recognised; methyl violet should therefore not be omitted. *Br. suis* grows in the presence of thionin only; strains of Danish origin are more sensitive to all dyes than American strains, but they are also less sensitive to thionin than to the other dyes. The dye-sensitivity test is of considerable value for the differentiation of brucella species if it is used, as Topley and Wilson suggest, only in conjunction with the other available tests.

The deep-strip method of applying selective bacteriostatic agents can be applied to the isolation and study of other bacterial groups, but for such purposes it probably has no advantage over the more orthodox methods.

### Summary

A simple method of testing the bacteriostatic effect of dyes on organisms of the genus *Brucella* is described, the dyes being incorporated in filter-paper strips embedded in liver-agar plates. The method has proved of value in the differentiation of brucella species.

I am indebted to Mr W. T. Bush for the preparation of the photograph.

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SQUAMOUS METAPLASIA IN THE PROSTATE AS A RESULT OF  
STILBÆSTROL THERAPY

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(PLATES LIX AND LX)

The following report describes the effect of stilbæstrol on the normal human prostate, the drug having been administered because of suspected prostatic cancer.

*Case history*

The patient, J. B., age 39, first came under observation in July 1942 with a gradual protrusion of the right eye. This was thought to be due to intra-orbital neoplasm and radiotherapy was undertaken, but it soon became obvious that no effect was being produced. In October 1944 the site was investigated surgically by Mr Norman Dott, who removed a tumour from the orbit. This proved histologically to be a carcinoma of large clear-cell type, with no obvious indication of the site of the primary growth. In March 1946 the patient returned to Dundee Royal Infirmary and X-ray examination now revealed tumour deposits in the skeleton. He looked much older and thinner than when he was first seen. At this time the serum acid phosphatase was 10 units (normal 2.3 units). Prostatic cancer was suspected and, although clinical examination of the prostate did not support this diagnosis, treatment with stilbæstrol was started. After three months' treatment there was striking improvement in his general health with a gain in weight of five stones; subsequently, however, his condition deteriorated and death occurred one year after the beginning of hormone therapy.

Three weeks before death (April 1947) the patient complained of persistent vomiting, occipital headache and pain in the right eye. Examination of the cerebrospinal fluid showed proteins slightly raised (50 mg./100 c.c.), sugar normal and chlorides normal (705 mg./100 c.c.). There was no pleocytosis and the Lange reaction was negative.

On 17/4/47, three days before death, the acid phosphatase level was normal (3 units). During the first three months of treatment the records show that the patient had taken ninety-six 5-mg. tablets of stilbæstrol; the dosage was then reduced to one tablet every second day, but the patient failed to report regularly and there is no certainty that he continued with this treatment.

*Summary of post-mortem findings*

The body showed marked pallor and there appeared to have been a somewhat rapid loss of subcutaneous fat. There was obvious hypertrophy of the breasts. Cancer deposits were present in the lungs, suprarenals, pituitary, cerebrum and vertebral bodies, on the inner surface of the dura mater in all the basal fossæ and along both sides of the superior longitudinal sinus. A small plaque of carcinoma in a bronchus, confirmed microscopically, was considered to be the primary growth.

The prostate as a whole was not obviously enlarged, although on section there was generalised cystic change. The colliculus seminalis, however, was obviously hypertrophied and had the flat white appearance associated with sodden squamous epithelium.

## SQUAMOUS METAPLASIA IN PROSTATE



FIG. 1.—From a deeper portion of the prostate, showing partial squamous change in the epithelium. The corpora amyloacea are of the soft non-senile type. Hæmalum and eosin.  $\times 170$ .

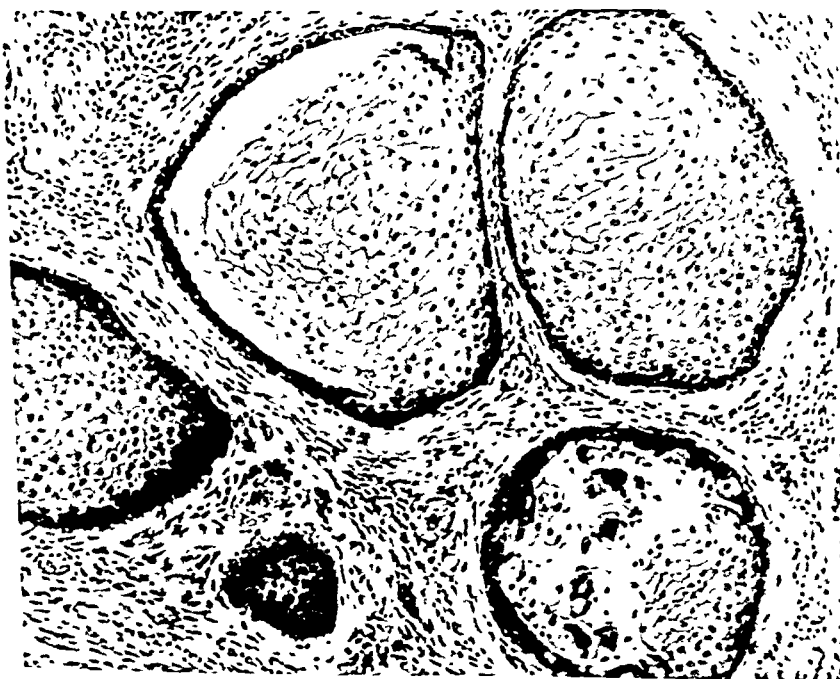


FIG. 2.—This field, from the deeper part of the uterine, shows the details of the squamous metaplasia and hyperplasia. Hæmalum and eosin.  $\times 120$ .

SQUAMOUS METAPLASIA IN THE PROSTATE AS A RESULT OF  
STILBÆSTROL THERAPY

ALEXANDER INGLIS

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(PLATES LIX AND LX)

The following report describes the effect of stilbæstrol on the normal human prostate, the drug having been administered because of suspected prostatic cancer.

*Case history*

The patient, J. B., age 30, first came under observation in July 1942 with a gradual protrusion of the right eye. This was thought to be due to intra-orbital neoplasm and radiotherapy was undertaken, but it soon became obvious that no effect was being produced. In October 1944 the site was investigated surgically by Mr Norman Dott, who removed a tumour from the orbit. This proved histologically to be a carcinoma of large clear-cell type, with no obvious indication of the site of the primary growth. In March 1946 the patient returned to Dundee Royal Infirmary and X-ray examination now revealed tumour deposits in the skeleton. He looked much older and thinner than when he was first seen. At this time the serum acid phosphatase was 10 units (normal 2-3 units). Prostatic cancer was suspected and, although clinical examination of the prostate did not support this diagnosis, treatment with stilbæstrol was started. After three months' treatment there was striking improvement in his general health with a gain in weight of five stones; subsequently, however, his condition deteriorated and death occurred one year after the beginning of hormone therapy.

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The body showed marked pallor and there appeared to have been a somewhat rapid loss of subcutaneous fat. There was obvious hypertrophy of the breasts. Cancer deposits were present in the lungs, suprarenals, pituitary, cerebrum and vertebral bodies, on the inner surface of the dura mater in all the basal fossæ and along both sides of the superior longitudinal sinus. A small plaque of carcinoma in a bronchus, confirmed microscopically, was considered to be the primary growth.

The prostate as a whole was not obviously enlarged, although on section there was generalised cystic change. The colliculus seminalis, however, was obviously hypertrophied and had the flat white appearance associated with sodden squamous epithelium.

SQUAMOUS METAPLASIA IN PROSTATE

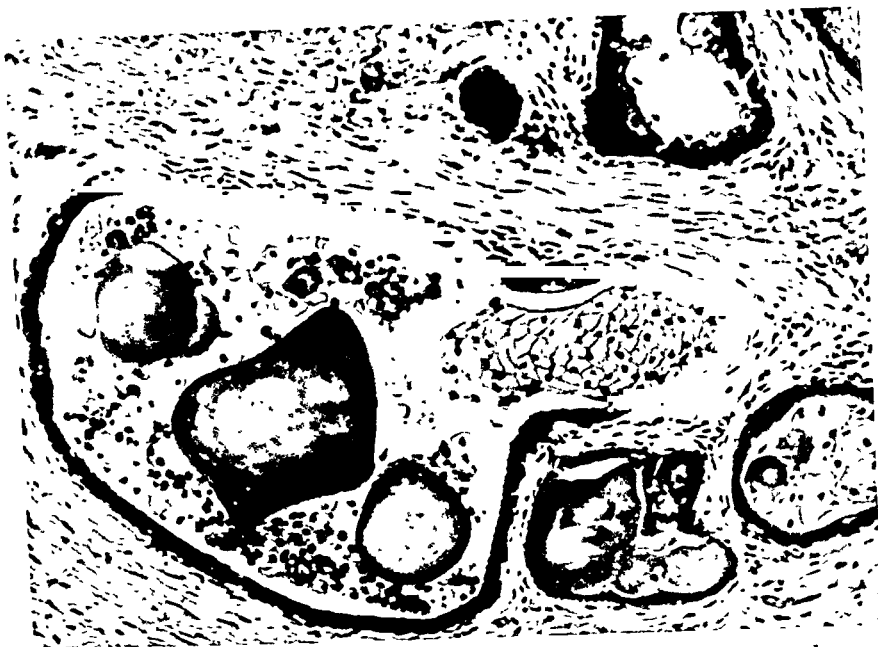


FIG. 1.—From a deeper portion of the prostate, showing partial squamous change in the epithelium. The corpora amylacea are of the soft non-senile type. Hæmalum and eosin.  $\times 170$ .

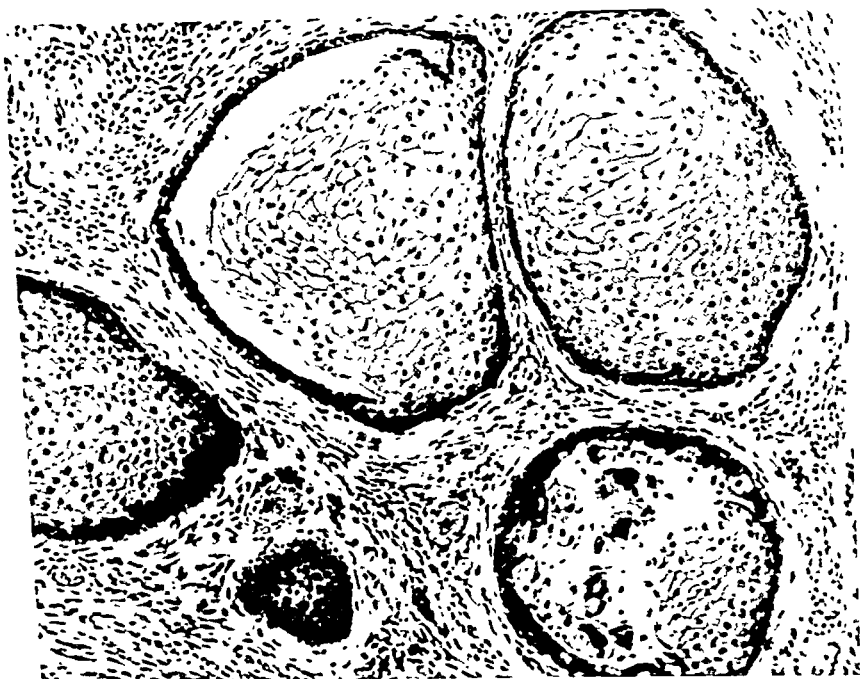


FIG. 2.—This field, from the deeper part of the utricle, shows the details of the squamous metaplasia and hyperplasia. Hæmalum and eosin.  $\times 120$ .



SQUAMOUS METAPLASIA IN PROSTATE



FIG. 3.—Colliculus seminalis (verumontanum), showing gross squamous hyperplasia of the overlying urethral epithelium. The utricle, lying in the centre of the projecting colliculus, shows not only metaplasia to squamous epithelium but extreme distension of its ducts by the epithelial hyperplasia. Hæmalum alone.  $\times 20$ .



Microscopically, squamous metaplasia of the glandular epithelium is seen in all parts of the prostate (figs. 1 and 2), especially its medial and postero-superior parts. The enlarged colliculus seminalis shows extreme squamous metaplasia within the utricle and hyperplasia of the overlying urethral epithelium (fig. 3). The mucosa of the rest of the prostatic urethra shows a similar although less advanced change. The ejaculatory ducts are not altered. Nowhere in the prostate is there any evidence of neoplasm or of chronic inflammatory reaction.

### *Discussion*

Squamous metaplasia is known to occur in the prostate and related glands of certain animals subjected to the effects of oestrogenic hormones (Zuckerman, 1935-36) and their synthetic substitutes (Burrows, 1935a). The present case shows that a synthetic oestrogen can produce changes in the human prostate which, when compared with the effects described in experimental animals, show a remarkable similarity, both in the nature and in the distribution of the hyperplasia and metaplasia.

In the early days of the experimental work with oestrone, its administration to normal male rats was shown to cause a decrease in weight of the prostate without any significant change in histological appearance, whereas administration to castrated rats elicited hypertrophy of the fibro-muscular layers of the dorsal lobe with obvious metaplasia and hyperplasia of the epithelium (Korenchevsky and Dennison, 1935). Thus it looked as if hyperplasia could occur only in the absence of testicular secretion, but it was soon found to take place in non-castrates if the dosage was maintained for a sufficient time (Parkes and Zuckerman, 1935). Subsequently, Burrows (1935a) showed that any substance pharmacologically related to oestrone could, with sufficient dosage, bring about prostatic metaplasia in animals.

The oestrogenic activity resident in an alcoholic extract of pig testis (Emmens and Parkes, 1938) has also been shown experimentally to produce metaplasia in the prostate, an effect which is inhibited by testosterone propionate. In general, the experimental work shows that oestrogenic substances, whatever their origin, can produce metaplastic changes in the prostate of rodents, dogs and apes, provided the dosage is sufficiently high or sufficiently prolonged, and can be effective in much lower doses if the counteracting male hormone is reduced by surgical interference.

On the strength of these conclusions, experimental workers have suggested that benign enlargement of the human prostate may in like manner be due to an imbalance of the sexual hormones, and Emmens and Parkes (p. 279) write:—"The fact that prostatic enlargement is typically a disease of senescence gave rise to the suggestion that it originated in testicular imbalance or deficiency and that male hormone therapy might be beneficial. The subsequent discovery that prostatic changes somewhat similar to those seen in man could be produced in laboratory animals by the administration of oestrone made it possible that the causative disturbance was an increase, during senescence, of the ambisexual oestrogenic activity of the testis, either an absolute increase or an increase relative to androgenic activity".

A possible presumption underlying these statements is that the histological differences between the prostate of the experimental animal altered by oestrogens and the human prostate showing benign enlargement are due to difference in species, the stimulating agent being the same. The present case, however, shows that the changes produced by stilboestrol in the human prostate closely resemble those seen in the experimental animal and are quite unlike the picture of the benign enlargement as ordinarily seen in man.

It is of course still possible that both in the present case and in the experimental animal we are observing a change brought about much more rapidly



than the rate of change which eventually produces ordinary benign enlargement. From the work of Parkes and Zuckerman it appears that duration of treatment bears a direct relationship to the degree of hyperplasia in the animal prostate. Burrows (1935*b*) has shown that the metaplasia in the experimental animal is reversible, and it may well be that benign enlargement of the human prostate is the end-result of frequent slight to-and-fro changes of much less degree than those found in the present case. It must be added, however, that this patient had received no oestrogenic therapy for at least three weeks before death, and probably for longer; yet the metaplastic changes are still almost as extreme as those demonstrated in the heavily dosed experimental animals.

It seems likely that the present widespread use of synthetic oestrogen will provide further evidence as to the sensitivity of the human prostate to those substances and may reveal whether or not the metaplastic changes are as readily reversible as in the rodent.

### Summary

A man of 39 with a latent cancer of bronchus and widespread metastases was treated with stilboestrol on a clinical diagnosis of prostatic cancer. The prostate, which contained no neoplasm, shows extreme squamous metaplasia and hyperplasia of its epithelium. These changes mimic closely in type and distribution those seen in the prostate of the experimental animal subjected to oestrogenic substances. The histological picture is quite unlike that of ordinary benign enlargement of the prostate.

I am grateful to Mr Norman Dott and Colonel W. F. Harvey for the clinical and histological data, which they so readily provided, relating to the patient's operation in Edinburgh, and to Professor Lendrum for his help.

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616 . 71—018 . 46—072 . 5—092 . 9

### MARROW BIOPSY IN LABORATORY ANIMALS

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During experimental observations on animals bone-marrow studies are often wanted. Preparations made after the animal has been killed are unsatisfactory unless fixed immediately after death, and it is often undesirable to kill the animal. To overcome these difficulties and permit of repeated observations on the same animal, a technique was devised which is applicable to rabbits, rats, guinea-pigs and mice, and requires no special apparatus, although a dental drill is useful.

Under general anaesthesia a small incision is made over the crest of the ilium posteriorly and the posterior superior spine exposed. If a dental drill is available an opening is cut on the lateral aspect of the spine down to the endosteum sufficiently large to permit of the introduction of a no. iii serum needle into the medulla. A small quantity of marrow fluid is gently aspirated into the needle by means of a syringe, the needle is withdrawn and its contents are expressed on to slides. Smears are prepared according to the method of Davidson, Davis and Innes (1943); six preparations can usually be made from one biopsy. The wound is closed by a single stitch.

In the absence of a dental drill, in guinea-pigs, rats and mice, the cortex over the lateral aspect of the posterior superior spine can readily be shaved away with a sharp scalpel. In rabbits this procedure is difficult, but a suitable opening can be made in the cortex of the external surface of the ilium just below the iliac crest by rotating the point of a narrow-bladed tenotomy knife against it. To prevent its being blocked the needle should be introduced into the medulla with the bevel towards the ilium. After 14 days there is no difficulty in obtaining further satisfactory preparations from the same bone through the same opening, the needle being introduced in a different direction. Employment of the contra-lateral bone permits observations at weekly intervals.

If there is delayed healing or other local complication, or if more frequent biopsies are desired, the femur may be used as an alternative site. It is less easy of approach, however, and requires a dental drill. Under general anaesthesia a small incision is made over the lateral aspect of the proximal third of the femur. The periosteum is stripped and an oblique opening is cut in the cortex down to the endosteum, permitting the introduction of a no. iii serum needle at an acute angle to the shaft. The subsequent stages are as described above. This method is simpler than that described by Vigran (1947) for obtaining marrow from the femur of the rat.

With this technique there is no difficulty in making preparations which stain satisfactorily with Leishman and May-Grünwald stains and are comparable in quality with those obtained from human sternal punctures.

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#### OBSERVATIONS ON FRAGMENTS OF GASTRIC MUCOUS MEMBRANE FOUND IN ASPIRATED RESTING GASTRIC JUICE

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(PLATES LXI-LXIII)

Einhorn (1894) described seven cases of what he called erosions of the stomach, all of which had similar symptoms—anorexia, weakness, loss of weight and epigastric pain. In these cases, gentle gastric lavage produced in the wash water up to four pieces of red mucous membrane varying in size from a pin-head to a centimetre in length. Histological sections of these pieces showed glandular

tissue into which bleeding had occurred, but no detailed microscopic examination appears to have been made. The subjects were males ranging from 26 to 36 years of age; four had no free hydrochloric acid in the gastric juice, two had hypochlorhydria and one hyperchlorhydria. As to the possibility of removal of these pieces from the stomach by trauma from the tube, Einhorn says "we have not to deal here with an incidental lesion caused by the tube, for, on the one hand, the lavage has been performed without any aspiration and by means of a soft tube; on the other hand, one could not observe in a casual lesion that constancy which is found here". Einhorn (1899) published notes of thirteen further cases, of which four were women, all of whom showed similar findings; their ages ranged from 23 to 60.

Although Einhorn's observations are mentioned in later works, as by Bolton (1913, p. 4) and, recently, photomicrographs of similar pieces of mucous membrane have appeared in textbooks, *e.g.* Bockus (1943, vol. i, p. 301), no extensive investigation of these pieces seems to have been made.

One of us (Hawksley, 1939), unaware of Einhorn's work, described a series of 200 cases subjected to gastroscopy for various gastric symptoms from more than 10 per cent. of whom one or more pieces of mucous membrane similar to those of Einhorn were found in the resting juice aspirated immediately before gastroscopy. Though aspiration and not lavage was used for removal of the juice, it appeared probable that these pieces of tissue were already present in the juice and were not detached by trauma for the following reasons:—(1) Aspiration was fairly gentle and the tube was of soft rubber. (2) No hæmorrhage occurred, as no blood was present in the resting juice and there was no trickling from the interior of the stomach on subsequent gastroscopy some five minutes later. (3) Attempts at removal of similar pieces from freshly resected stomachs either by suction along similar lines or even by finger-nail scratching were unsuccessful.

The pieces were obtained from cases subsequently diagnosed as gastric ulcer, duodenal ulcer, gastritis and carcinoma, and there was nothing to associate them specially with any particular disease. Since the resumption, two years ago, of gastroscopy, using a similar technique, these pieces have continued to be recovered in about 10 per cent. of cases, but as nearly all were cases of ulcer, no idea of their frequency in other diseases of the stomach has been obtained.

### *Histology*

Ranging in size from a pin's head to a centimetre in length, as did Einhorn's specimens, these pieces (two of which are shown in fig. 1) have been subjected to histological examination. The material obtained was immediately fixed in 10 per cent. formol-saline and the whole piece of mucosa in each case embedded in paraffin. Sections were stained with hæmatoxylin and eosin and hæmatoxylin and van Gieson.

A microscopical study showed a varying but usually considerable degree of gastritis and it was possible to recognise in this material all the histological types of gastritis described by Faber (1935, pp. 26-50). We have not, however, observed the pseudopyloric glands nor (by the appropriate staining methods) either argentaffine or Paneth cells (Magnus, 1937).

The specimens fall into two main categories—those showing acute, and those showing chronic gastritis. In the acute gastritic cases the mucosa is hyperæmic and oedematous, with hæmorrhages in the subepithelial and interglandular tissue. There is marked diffuse polymorphonuclear-cell infiltration, including invasion of the epithelium of both glands and crypts. The epithelial cells in some cases show vacuoles containing leucocytes (fig. 2). In the chronic gastritic cases there is marked variability in the histological features. A large proportion show intense infiltration of the interglandular tissue with plasma

GASTRIC MUCOSA IN ASPIRATED GASTRIC JUICE



FIG. 1.—Two large pieces of gastric mucous membrane found in aspirated resting juice.  $\times 5$ .



FIG. 3.—Follicular gastritis. H. and E.  $\times 150$ .

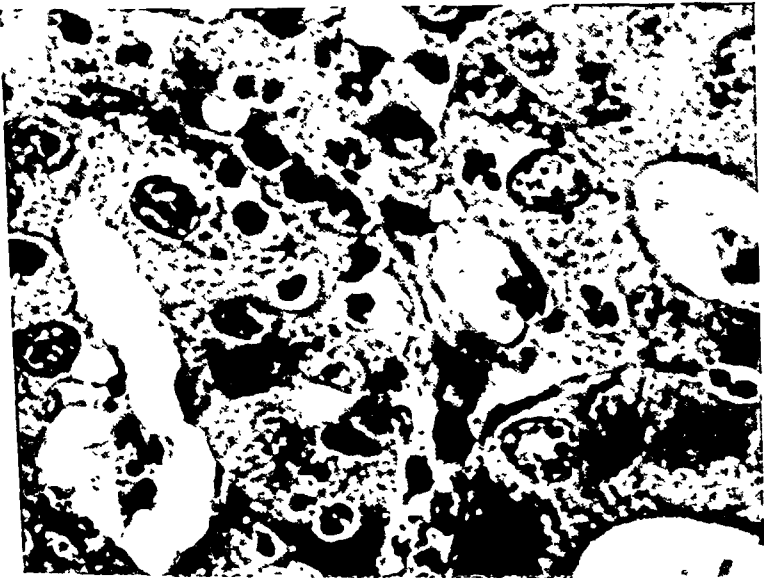


FIG. 2.—Acute gastritis showing polymorphs, some of which are in epithelial cells. H. and E.  $\times 800$ .



GASTRIC MUCOSA IN ASPIRATED GASTRIC JUICE



FIG. 4.—Atrophic gastritis. H. and E.  $\times 80$ .

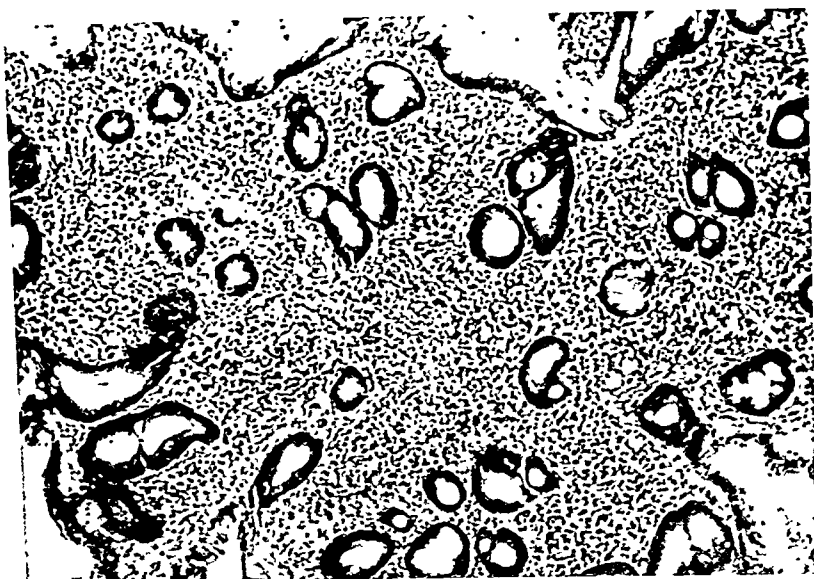


FIG. 5.—Cystic gastritis. H. and E.  $\times 90$ .

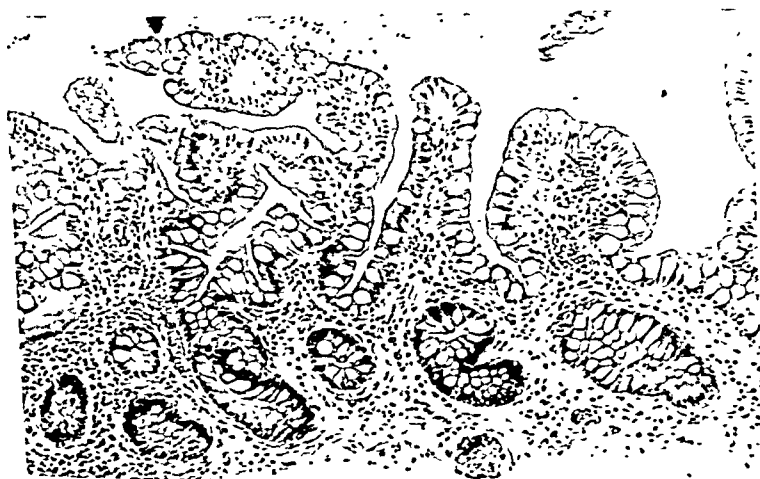


FIG. 6.—Intestinal heterotopia. H. and E.  $\times 95$ .



GASTRIC MUCOSA IN ASPIRATED GASTRIC JUICE



FIG. 7.—Strands of smooth muscle irregularly dispersed amidst glandular tissue.  
H. and E.  $\times 120$ .



FIG. 8.—Carcinomatous infiltration. H. and E.  $\times 95$ .





cells and lymphocytes: others present evidence of degeneration, atrophy and faulty regeneration of the epithelium, while a few show aggregations of lymphocytes, giving rise to the appearance of follicle formation—follicular gastritis (fig. 3). In chronic atrophic gastritis, cystic changes are not infrequent and there is often a patchy replacement of the gastric-type mucosa by a mucous membrane of intestinal type—superficial intestinal heterotopia (Taylor, 1927).

In atrophic gastritis the most striking change is the reduction in number of the secreting-glands, with relative excess of interstitial tissue, which is diffusely and sometimes intensely infiltrated with lymphocytes and plasma cells (fig. 4). Chronic cystic gastritis is characterised by the presence of a large number of cystic glands of various sizes, the intervening tissue being again infiltrated with plasma cells and lymphocytes (fig. 5). In atrophic gastritis with intestinal heterotopia the mucosal surface is covered, often over large areas, with cylindrical epithelium containing many goblet cells; indeed the mucosa as a whole is indistinguishable from that of the small intestine (fig. 6). One case of chronic gastritis showed conspicuous strands of smooth muscle between the glands (fig. 7). The contraction of such fibres might well have the effect of increasing the mucosal rugosity.

In another (single) case we observed carcinomatous infiltration (fig. 8), the rest of the fragment showing marked gastritic changes. Bolton (1913, p. 35) observed superficial acute ulceration occurring over areas of mucous membrane infiltrated with growth. Such a process might well account for the presence of pieces of detached cancerously-infiltrated mucosa.

#### Discussion

Nearly all the fragments examined have shown considerable and most of them severe degrees of gastritic change, both acute and chronic.

It remains to be decided whether or not these pieces separate naturally or are pulled off in the process of aspiration. Reasons are given by Einhorn and by us for believing that the former mechanism is the more likely. Against this is the fact that our specimens do not show the partial digestion that might be expected if they had been separated into the stomach for more than a few minutes. It is possible that many such separations of small portions of mucosa do occur in subjects with gastritis, but that digestion removes them so quickly that they are only easily found when the stomach is in a resting phase and the digestive secretions in abeyance. This would have been the case with all our subjects, who were examined in the early morning after fasting overnight. It is also possible that if gentle suction can pull these pieces off without production of hæmorrhage into the stomach, they represent pieces that are about to separate or to be digested *in situ* as soon as peptic secretion occurs. Bolton (1913, p. 30) has observed such partially digested pieces of mucosa still *in situ* where erosions or acute ulcers are forming. The small areas covered by such devitalised or partially devitalised pieces of tissue would form what geologists would call "lines of easy cleavage", where slight suction might well be enough to cause their detachment. According to Kenamore *et al.* (1946) biopsy of living gastric mucosa usually causes recognisable bleeding.

Our findings thus lead us to believe that Einhorn may be right in discounting trauma, but that, as an alternative, these pieces may be readily detached owing to their devitalised state. In any case they present a means of study of the changes of gastritis in detached portions of mucosa, and our own material demonstrates the severe degree of gastritis that may be present.

#### Summary

1. The recovery of mucosal fragments from the wash water after gastric lavage is recorded; no detailed histological description of such fragments has been found in the literature.

2. We have found these fragments in the resting gastric juice, obtained by aspiration prior to gastroscopy, in 10 per cent. of cases.

3. Nearly all showed a considerable and most a severe degree of gastritic change. In one case fragments of carcinomatous tissue were present.

4. The mechanism of separation of these fragments is discussed and it is suggested that trauma is not the principal factor. They may, however, be more readily detached owing to their diseased state.

5. The microscopic examination of these fragments provides a new method of studying the changes in gastritis.

Our grateful thanks are due to Professor G. R. Cameron, F.R.S., Director of the Graham Laboratories, University College Hospital, for facilities, to Mr J. H. Bayley for his great help in the preparation of sections, and to Mr K. S. Macdonald for the photomicrographs.

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615 . 7 : 576 . 893 . 161 . 13 (*Trypanosoma congolense*)

THE CHEMOTHERAPY OF *TRYPANOSOMA CONGOLENSE* INFECTION WITH PHENANTHRIDINIUM COMPOUNDS ETC.\*

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Experimental *Trypanosoma congolense* infections in mice are powerfully influenced by certain phenanthridinium compounds prepared by Morgan and Walls, whereas they are extremely refractory to drugs which are actively trypanocidal for *T. brucei* (Browning *et al.*, 1938, 1940). In order to obtain consistent results which permit a reliable comparison of the therapeutic effects of different drugs, it is necessary to maintain strains of the trypanosomes by passing the infection to fresh animals as soon as the parasites become abundant in the blood, *i.e.* at "acme", and not to wait till a subacute or chronic stage is reached. Both the immunological properties and the sensitiveness to chemotherapeutic agents are preserved unchanged, apparently indefinitely, by such an acme strain (Browning and Calver, 1943; Calver, 1945). In contrast

\* Work done with the support of the Medical Research Council.

to *T. brucei*, *T. congolense* infections are more sensitive to phenanthridinium drugs at the acme stage than earlier, before trypanosomes are numerous in the blood. This fact suggests that the immunity reaction following destruction of the organisms under the influence of the drug plays a significant part in conducing to cure. At the subacute or chronic stage the infection is less responsive than at acme.

*Therapeutic efficiency of different phenanthridinium compounds  
and other drugs*

Since the work with 7 amino-9 (*p*-amino phenyl)-10-methyl phenanthridinium chloride (no. 897) a series of analogous compounds have been synthesised by Walls (1947). Of these 2:7 diamino-9 phenyl-10 methyl phenanthridinium bromide (no. 1553, "dimidium bromide") showed the greatest therapeutic effect, as measured by the ratio of the highest tolerated dose to the minimum curative dose. The table gives the results, also those with 4:4'-diamidino

TABLE

*Results of treatment at "acme" of mice infected with T. congolense*

Drug *	Dose as a fraction of the maximum tolerated dose	No. cured/no. treated in the case of strain †		
		I	II	III
No. 897 (1 mg.) . . .	1/5-1/10	...	15/22	23/38
	1/15	...	34/75	0/4
	1/100	23/41	...	...
No. 1553 (1 mg.) . . .	1/40-1/50	...	...	23/37
	1/60-1/90	...	...	0/7
	1/100	...	25/28	...
	1/200	...	7/17	...
	1/400	14/24	...	...
Diamidino compound (2 mg.) .	1/5	...	10/12	...
	1/8	...	...	20/25
	1/10	22/27	5/26	...
	1/12	...	...	0/17
	1/20	8/28	...	...
	1/30	2/24	...	...

\* The maximum tolerated dose in mg./20 g. body weight is given in brackets; the dose is injected subcutaneously in a volume of 1 c.c.

† Strains I and II are those referred to by Browning and Calver. For strain III, originally isolated by Mr J. B. Randall, Entebbe, Uganda, we are indebted to Imperial Chemical Industries, Biological Department, Manchester. Its virulence is intermediate between that of the other two strains, since after acme the trypanosomes remain abundant in the blood of two-thirds of the mice until they die, while parasites disappear temporarily in the remainder. A "chronic" strain derived from strain III retained its altered serological characters after 25 passages at acme over 6 months.

dimethyl stilbene (Fulton and Yorke, 1943). The organic antimony compound "stibophen" (for which we are indebted to Messrs Burroughs Wellcome) was examined, but half the tolerated dose was not certainly curative for strain III. The variation in sensitiveness of the different strains is striking. With the phenanthridinium drugs the ratio of effectiveness for strains I, II and III is approximately 9:3:1, whereas with the diamidino compound the extreme ratio does not exceed 2:1. In general, the latter drug is also the least active. Wien's (1946) results with no. 1553 confirm ours; but Fulton and Yorke, and Wien consider no. 897 and the diamidino compound practically equal. It appears that these workers treated infected animals earlier, when parasites

then embedding the explants on the upper surfaces of the slips. The fluid medium is then added and the flasks incubated. This method permits of the removal of one slip at a time for high-power examination of the growth at different stages of development.

The testicular tissue was first minced finely with scissors and the fragments were then placed on coverslips on the bottom of Carrel flasks (internal diameter 3 cm.) in a small drop of chick plasma which quickly clotted. To each flask 2.4 ml. of fluid medium were added. This medium consisted of

Tyrode's solution . . . . .	70 parts
Chick embryo extract . . . . .	5 "
Rabbit serum . . . . .	25 "
Phenol red . . . . .	a trace

Care was taken to adjust the pH of the fluid medium to 7.4, at which optimal growth of testicular tissue is obtained. This was done by bubbling through the medium a mixture of oxygen and 5 per cent. carbon dioxide and matching the colour against a set of standard phenol red solutions.

The fluid medium was drawn off and renewed on the 5th and 10th days, and at the same time one or more cultures on their coverslips were removed for examination. These were fixed in methyl alcohol and stained by Giemsa's method. In tissue infected with *Treponema pallidum*, the specimens were stained also by Levaditi's method. In addition fresh wet preparations were examined with the phase-contrast microscope, while the fluid medium was tested for infectivity by intratesticular inoculation into healthy rabbits.

#### *Cultures of normal rabbit testis*

*Newly-born rabbits.* Each testis provided about 8 explants. Growth usually began early and on the second day a halo of cells was visible around the explant. In most cases fibroblasts then grew out from opposite poles of the explant, forming two chains of cells which later converged and met, thus forming a ring of new tissue with a diameter of 2.4 mm. This stage of development was usually reached about the fifth day, and occurred in nearly 60 per cent. of preparations: in the remainder there was complete failure of growth after the second day. By the fifth day also epithelial cells were easily visible, growing as a solid sheet from the edge of the explant within the circle of fibroblastic growth. Macrophages were found scattered both inside and outside the circular area. Fig. 1 shows a corner of one explant and illustrates well the various types of cell which grew.

*Young adult rabbits.* The testis, in its capsule, was removed aseptically from an anaesthetised animal and, after decapsulation, was minced and explanted as described. Growth did not occur so frequently nor was it so vigorous as with the material from new-born rabbits. When it did occur, however, it was of the same type, and epithelial proliferation was observed in about 20 per cent. of preparations, each experiment being carried out with at least 80 explants.

#### *Cultures of rabbit testis previously infected in vivo with 'Treponema pallidum'*

I am indebted to Dr Selbie of the Middlesex Hospital, London, for providing me with a strain of *Treponema pallidum* which had previously been isolated from a human case of syphilis and was maintained in rabbits by intratesticular inoculation. The testis was removed about 6 weeks after inoculation, when the organ was swollen and hard, and before ulceration had begun. After ulceration has started there is grave danger of carrying over secondary pyogenic invaders into the tissue-culture flasks.

CULTURE OF *TREPONEMA PALLIDUM*



FIG. 1.—Corner of explant of normal rabbit testis showing (a) part of fibroblastic ring of cells, (b) sheet of epithelium, (c) scattered macrophages.  $\times 65$ .



FIG. 2.—Giant cell with spongy vacuolated cytoplasm and 7 nuclei.  $\times 1250$ .

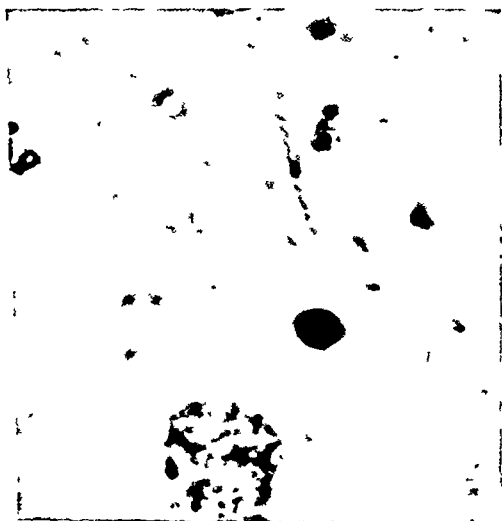


FIG. 3.—Phase-contrast photomicrograph of culture showing *Treponema pallidum*.  $\times 1800$ .



In each of three experiments at least 80 explants were set up; in two experiments healthy growth of only three explants was observed; in the third experiment only two explants grew well. Most of the others did not grow after the second day. They exhibited no more than a few sprouts of fibroblasts and some scattered macrophages and there was no evidence whatever of epithelial proliferation. In the explants which grew normally the usual arrangement of fibroblasts, macrophages and epithelium was maintained. In addition there were numerous multinucleated giant cells with spongy vacuolated cytoplasm and seven or more nuclei (fig. 2). Although this type of cell is common in many other kinds of tissue culture, it was seldom observed in the cultures of normal testis. It is possible, therefore, that these cells, which resemble the giant cells commonly associated with granulomatous inflammatory lesions, may represent a tissue response to the invasion by treponemata.

Examination of fresh wet preparations with the phase-contrast microscope showed living organisms in two cases, in cultures removed on the 5th and 7th day respectively (fig. 3). In both these preparations growth was healthy and reasonably vigorous. No organisms were ever found in cultures which had not grown.

After 10 days' incubation the fluid medium from the flasks in which growth had occurred was pooled and inoculated intra-testicularly into a healthy rabbit. This animal later developed a typical chancre in which *Treponema pallidum* was easily demonstrated. Similar inoculation of the fluid from the flasks in which growth had not taken place did not prove infective.

No organisms were ever demonstrated in cultures stained by Levaditi's method.

#### Discussion

The most widely used of the large number of methods of culture of *Treponema pallidum* previously investigated has probably been that developed and described by Eagle (1940). In this case the organisms survived only for a few hours. Survival in other types of fluid media is also generally limited to a few hours, although survival for longer periods has occasionally been reported. Using a centrifuged suspension of rabbit testis plus rabbit serum, Eagle (1938) was able to maintain the cultures for at least 48 hours.

The method of culturing *Treponema pallidum* now described has enabled the organisms to survive for periods up to 10 days. Survival was apparently dependent upon the presence of living testicular cellular elements. Unfortunately successful cultures were obtained only in a small proportion of preparations, which reduces the general usefulness of the method considerably. But this success, though limited, seems worth recording and it is to be hoped that further work may lead to improved results.

#### Summary

The growth of testicular epithelium *in vitro* has been confirmed, and successful cultures were obtained in most preparations in which the testes were taken from newly born rabbits. Cultures were also successful, although in a smaller proportion of cases, when the testes of young adult rabbits were used.

The same technique was used for the *in-vitro* growth of adult rabbit testis previously infected *in vivo* with *Treponema pallidum*. In some of the preparations survival of the organism for periods up to 10 days was demonstrated.

I am indebted to Miss J. Barten for technical assistance and to Mr F. Welsh, F.R.M.S., for the photomicrographs.



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# SIDEROCYTE COUNTS IN THE BLOOD OF NORMAL AND PREMATURE INFANTS

HELEN PAYLING WRIGHT and DENISE G. EDMONDS

*From the Obstetric Unit, University College Hospital, London*

The presence of "siderocytic" granules in the blood of flex-tailed mice and young rats was first shown by Gruneberg in 1941a. In the same year he reported their presence in small numbers in infants. The present short study was made, first, to expand the meagre data concerning these cells in normal and premature infants, and second, to correlate in this series the findings obtained with the two methods generally in use for the estimation of siderocytes—the prussian-blue staining method, and that of Case (1944-45) using aa' dipyriddy. No attempt is made here to elucidate the nature of the granules or to discuss whether the cell inclusions stained by the two methods are identical or not.

## Method

Fifty normal full-term infants and 7 premature infants were examined daily for the first 8 days of life. The degree of prematurity of the 7 children in the group was between 4 and 10 weeks. Parallel observations using the aa' dipyriddy staining technique and the prussian-blue method were made in 34 cases. Reticulocyte and siderocyte counts were compared daily in 25 infants.

Blood was obtained by heel prick and films were made for the siderocyte counts. The method of counting the reticulocytes was that of Price-Jones, Vaughan and Goddard (1935).

## Results

The daily counts together with their standard errors are set out in the table. The infants who showed "physiological jaundice" are included in the group as a whole but are also shown separately, as it was suggested by Case (1945) that the incidence of cell inclusions stained by his method increased in hæmolytic conditions.

From the table it can be seen that the percentage incidence of granule-containing cells stained by the two methods was very similar in the blood of new-born infants, and further, that during the first 8 days of life there was a steady, though small, decrease in the percentage of affected cells, which, by the end of the first week, approximates to that reported to be normal in adults, namely  $0.5 \pm 0.3$  per cent. (Case *et al.*, 1945).

In infants who exhibited "physiological jaundice" there appeared to be a rise in the siderocyte count from the second to the fourth day; this, however, was transitory, and there was a subsequent decrease so that by the eighth day their numbers were not significantly different from the non-jaundiced group.

TABLE

*Daily siderocyte and reticulocyte counts (per cent.) with the standard error of the means*

Day	0	1	2	3	4	5	6	7
Normal—								
aa' dipyrldyl .	1.10 ± 0.074	1.00 ± 0.056	1.00 ± 0.063	0.97 ± 0.070	0.90 ± 0.062	0.70 ± 0.061	0.94 ± 0.058	0.55 ± 0.037
Prussian blue .	1.10 ± 0.066	0.96 ± 0.041	0.92 ± 0.055	0.90 ± 0.043	0.80 ± 0.060	0.66 ± 0.046	0.60 ± 0.047	0.46 ± 0.060
Reticulocytes .	4.55 ± 0.268	3.93 ± 0.346	3.45 ± 0.391	3.00 ± 0.484	2.86 ± 0.490	0.79 ± 0.160	1.16 ± 0.340	0.57 ± 0.191
Jaundice—								
aa' dipyrldyl .	0.971 ± 0.110	0.96 ± 0.116	1.20 ± 0.128	1.40 ± 0.133	1.30 ± 0.188	0.93 ± 0.142	0.90 ± 0.101	0.69 ± 0.114
Premature—								
aa' dipyrldyl .	1.86 ± 0.267	2.15 ± 0.573	2.16 ± 0.512	2.35 ± 0.580	2.80 ± 0.189	1.48 ± 0.391	1.16 ± 0.265	1.10 ± 0.183
Reticulocytes .	5.60 ± 0.564	4.98 ± 0.608	3.87 ± 0.900	3.42 ± 0.701	4.03 ± 0.941	2.10 ± 0.756	1.33 ± 0.521	0.94 ± 0.494

There was a considerably higher percentage of granule-containing cells in the small group of premature infants, with the maximum occurring on the 4th day of life.

The reticulocyte counts call for no special comment since they followed the normal pattern for new-born infants in both the full-term and premature groups (Krumhhaar, 1932).

#### Discussion

There is at present considerable disagreement concerning the nature of the siderocytic granules stained by the aa' dipyrldyl and potassium ferrocyanide methods (McFadzean and Davis, 1947) and it is not proposed to discuss the controversial points at issue in this short note. It may be of interest, however, to point out certain features which have been found in the examination of the blood of new-born infants stained by these two methods and to comment on the present series of cases in the light of previous work by other investigators.

Grüneberg (1941b) first described iron-staining granules in circulating red blood cells. He reported that the full-term infant's blood contained about 0.25 per cent. of these cells, while in the 14-week foetus a count of 4.45 per cent. was obtained in the heart blood *post mortem*. In the present study, with both the staining methods used, a rather higher incidence was found in the blood of full-term infants. Corresponding to Grüneberg's findings on his single foetus, however, we have obtained a higher incidence (1.86 per cent., range 1.2-7.5 per cent.) in premature infants, using the aa' dipyrldyl technique. The incidence of siderocytes in the first day of life in the present series is about twice that reported as normal for adults by Case (1945), though by the end of a week the percentage approximated to his figure.

It is of interest that the infants who exhibited marked "physiological jaundice" exhibited a higher mean siderocyte percentage than that of the series as a whole, since a raised count has been reported in various hæmolytic conditions affecting adults (Doniach, Grüneberg and Pearson, 1943; Case, 1945; Pappenheimer *et al.*, 1945; McFadzean and Davis, 1947; Dacie and Doniach, 1947).

There appears to be no relationship between the incidence of granules stained by either the prussian-blue or the aa' dipyrldyl methods and the reticulocyte count. This finding confirms that of McFadzean and Davis and is contrary to

that reported by Case (1945), who also suggested that in certain conditions the reticulocyte/siderocyte index was of value in prognosis.

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## A CASE OF PRIMARY SARCOMA OF THE HEART

W. W. ADAMSON

*From the Royal Infirmary, Hull*

(PLATES LXV AND LXVI)

*Clinical history*

The patient, N. W., a gardener 24 years old, was admitted to hospital on 7.10.39 with a history of pain in the right axilla which had commenced two weeks previously. The pain was accentuated on taking a deep breath. There was also a history of his having had pain in the region of the stomach two weeks before admission, with vomiting. Symptoms of intestinal obstruction had been present for two days before admission. No faeces or flatus had been passed and there were colicky abdominal pains every few minutes, with repeated vomiting.

At operation on 8.10.39 a distended loop of ileum presented and a large ileal intussusception was reduced. A submucous tumour about the size of a hen's egg was palpable in the ileum, which was opened, and the tumour removed by diathermy.

As colicky abdominal pains continued to occur every few minutes and there was repeated vomiting, the wound was reopened on 20.10.39 and about five inches of jejunum were resected. Another nodule similar to the one already removed but about one-eighth its size was present in the resected piece of bowel.

After continuing to run an intermittent temperature, with vomiting of undigested food after meals, he suddenly complained of severe abdominal pain on 28.11.39, became collapsed, and died after four hours.

A CASE OF PRIMARY SARCOMA OF THE HEART

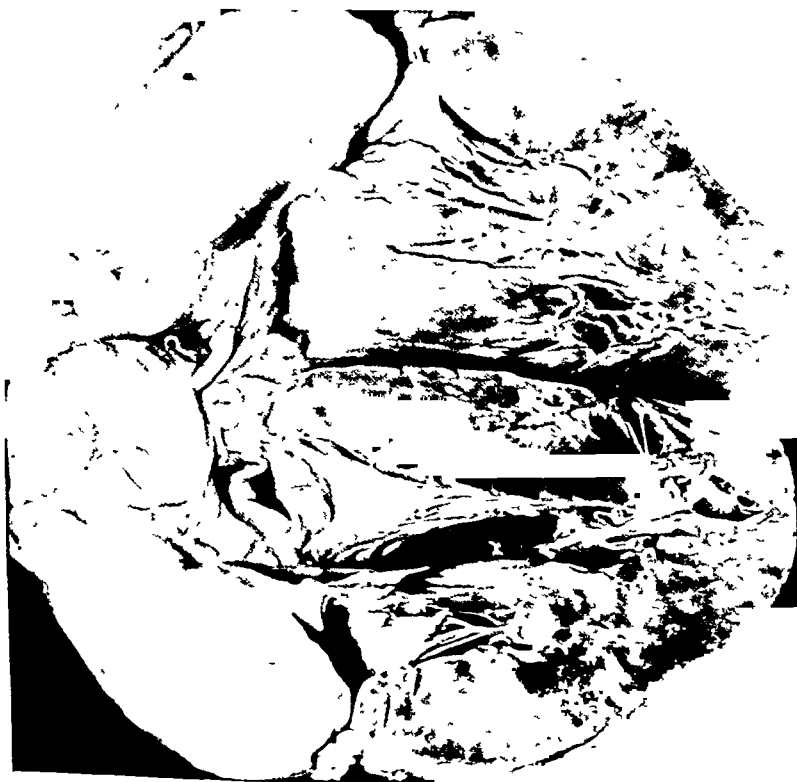


FIG. 1.—Main tumour in left atricle. The tumour has been divided in two and is seen situated above the mitral valve.



FIG. 2.—The specimen is here photographed from the left side to show the wall of the atricle turned downwards, allowing the secondary flat nodule on the wall to be seen.



*Post-mortem examination*

The body was much emaciated.

The left pleural cavity contained three ounces of yellowish fluid with some fibrinous material floating in it. The trachea, larynx and bronchi were normal. At the root of the right lung a hard mass of glands was found. The left auricle of the heart contained a large tumour which almost completely filled its cavity.

The mouth, œsophagus and stomach were normal. In the abdomen, acute peritonitis was present, with much fluid effusion and matting together of the coils of small intestine. There was a perforation in the upper part of the ileum. Many polypoidal tumours were present in the small intestine from the duodenum to the ileum and there were also two tumours in the ascending colon, three and eight inches respectively from the orifice of the appendix. The tumours ranged from 1 to 2.5 cm. in diameter.

The liver was very fatty. The gall bladder contained yellow bile. Two small black necrotic areas were present in the body of the pancreas. The kidneys showed cloudy swelling and fatty change. The suprarenals and bladder were normal. The retroperitoneal and mesenteric glands were greatly enlarged.

The brain and spinal cord were not examined.

*Heart.* The tumour in the left auricle (fig. 1), which almost completely filled its cavity, measured  $6.5 \times 5.0 \times 3.0$  cm. in diameter. Its surface, which was slightly nodular, was covered in its upper half by a reddish layer of fibrin. It was attached by a broad pedicle to the upper surface of the anterior mitral cusp and its base extended well on to the anterior wall of the auricle and backwards on to the inter-auricular wall. The colour of the cut surface varied from grey to white. A second nodule was present on the posterior and upper wall of the left auricle (fig. 2). This had a flat surface, nodular at its lower end, and formed a flat cap resting on the larger tumour. It measured  $4.0 \times 3.5 \times 1.0$  cm. in diameter.

*Histology*

Of the two tumours of the small intestine removed at operation the larger was a spindle-cell sarcoma, the smaller a mixed cell sarcoma (figs. 3 and 4). The tumour in the anterior wall of the left auricle was also of polymorphous-cell type. In it, cellular fibrous areas (fig. 5) were interspersed, and occasionally the structure was myxomatous. Necrotic cells and capillaries were present in some of the more fibrous areas. Spindle, round and giant cells were all present and mitotic figures were not infrequent. Bronchial and retroperitoneal glands, pancreas and kidney were examined microscopically for evidence of invasion but no tumour cells were found.

*Commentary*

Two methods of spread are illustrated in this case—by the blood stream and by direct contact. The flat nodule on the posterior wall of the left auricle, which appeared to be a secondary growth, showed a similar histological structure to the primary tumour of the anterior wall and was presumably due to contact infection. The secondary nodules in the intestine tended on the whole to be more cellular than the primary tumour in the heart. They were mainly in the area supplied by the superior mesenteric artery and were presumably embolic in origin.

Mahaim (1945) lists 87 cases of primary sarcoma of the heart, the order of frequency being spindle cell, round cell, giant cell and polymorphous cell. Among these there is only one example of secondary deposits in the intestines, namely case IV of Muller. Usually the secondary growths were in the lungs, liver and pericardium.

*Summary*

A case of primary sarcoma of the left auricle is described in which there was secondary extension by contact to another part of the auricular wall and by the blood stream to the duodenum, jejunum, ileum and ascending colon.

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A CASE OF PRIMARY SARCOMA OF THE HEART



FIG. 3.—Section of polypus of small intestine showing invasion of mucosa. H. & E.  $\times 120$ .

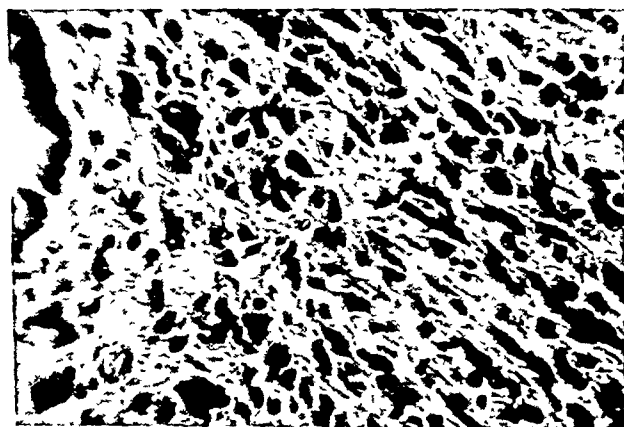


FIG. 4.—Same section (high power view).  $\times 450$ .

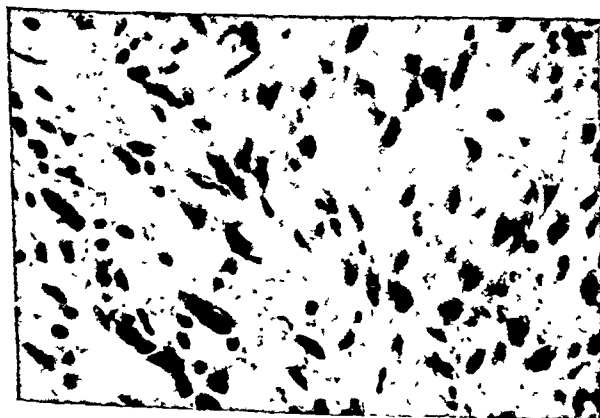


FIG. 5.—Tumour of the heart, showing one of the more cellular areas. H. & E.  $\times 450$ .





## BOOKS RECEIVED

### **The biology of melanomas**

Edited by ROY WALDO MINER, MYRON GORDON and LOTHAR SALIN.  
1948. Special Publications of the New York Academy of Sciences, vol. iv.  
Pp. xii and 466 ; 233 figs. on 107 plates (4 in colour) and 25 text figs. \$5.

This special publication of the New York Academy of Sciences consists of papers contributed by invited authors to a Conference, held in New York City in November 1946, on the biology of normal and atypical pigment-cell growth. The scope is thus rather wider than the title would suggest. The 26 papers presented range over the clinical and developmental, cytological and genetic, endocrinological and physiological fields, and finally the chemical and physical aspects of the problem.

"While much new information was presented at this conference, its special value was found in the fact that many details which were previously known only to specialists in their respective fields were disclosed to all workers. Yet it was obvious that it had merely exposed the many facets of an extremely complex field. Perhaps the greatest achievement was in bringing the subject of pigment cell biology into prominence". This extract from the foreword is endorsed by the reviewer.

It may be asked whether the general pathologist, or the clinician especially interested in melanotic tumours, is likely to find this book a valuable work of reference. Because it brings together, with good bibliographies, fields of work which the clinician and pathologist might otherwise overlook, it is certainly of real value. To the experimentalist in any branch of this field, it is a necessity.

It is only possible to mention here some of the papers presented. Masson confines himself to a description of the normal human pigment cell, though he calls on certain pathological observations which have helped in the understanding of its character and function. Pack accepts a neurogenic origin for the melanotic tumours of man ; he discusses their clinical aspects and advocates the surgical excision of both benign and malignant melanomas. For the latter, excision of the primary tumour and dissection in continuity of metastatic deposits in regional lymph nodes is strongly advocated. Becker discusses pigment formation in the skin and agrees with Masson that the epidermic melanoblast is a cell distinct from the palisade basal cell. He then goes on to discuss (with good illustrations) pigmentary dermatoses in the human subject. Goldberg marshals the arguments for the mesodermal origin of melanoblasts, while Macklin discusses the genetic background of pigment-cell growth. Her bibliography is extensive. Algire and Legallais describe the growth and vascularisation of the two well-known transplantable mouse melanomas as seen by transparent culture-chamber technique. Sugiura used one of these tumours (Harding-Passey) for a very thorough investigation of the effects of numerous physical and chemical factors, with results which were largely negative. Du Shane, Grand and Cameron, Levine and others are concerned with the pigment mechanism and the melanotic tumours of fishes, amphibians and birds.

The illustrations are of rather uneven calibre, though many of them are excellent. Discussions on the papers are disappointingly brief. The bibliographies are extensive, especially those on the animal aspects of the subject.

**The 1947 year book of pathology and clinical pathology**

"Pathology", edited by HOWARD T. KARSNER and HERBERT Z. LUND;  
 "Clinical pathology", edited by ARTHUR HAWLEY SANFORD. 1948.  
 Chicago: The Year Book Publishers Inc. Pp. 558; 103 text figs. \$3.75.

"Conditions of the war and post-war period have not permitted publication of the Year Book of Pathology since 1941". The problem of selection from the extensive and important literature of this seven-year period was a difficult one, and it was decided to concentrate on the most recent articles as likely to offer the greatest value to readers. Three-fifths of the volume is devoted to pathology, the remainder to clinical pathology, including a 20-page section on parasitology. In addition to some hundreds of abstracts, several short "special articles" are contributed by various authors, namely "mechanism of invasiveness of cancer" by Dale R. Coman, "pathology of lymphatic and hemopoietic organs" by Edward B. Smith and R. Philip Custer, "correlation between vaginal smear and tissue diagnosis in 1045 operated gynecologic cases" by Malcolm S. Allan and Arthur T. Hertig, and "current problems on bone pathology" by Henry R. Jaffe.

Most of the articles summarised are American, but there are many from British (including Australian) journals, and a few each from Swiss, Scandinavian, French and other sources. Some of the abstracts extend to 6 or 8 pages. The topics selected cover a wide field and have been chosen with discrimination. Full subject and author indexes are provided.

Instead of the usual "blurb" on the jacket, a new terror is added to life in the form of a "Year Book Quiz". "The scores of significant world-wide advances detailed in this 1947 Year Book shed important light on the new methods and procedures in laboratory medicine. Test your familiarity with the current literature by trying the questionnaire that follows"! The few errors which we have detected include the mis-spelling of Hassall ("Hassal's corpuscles", p. 109). This may be regarded as excusable, since the same error has been perpetuated throughout the whole series of editions of that authoritative work, Schafer's *Essentials of histology*.

**Textbook of endocrinology**

By HANS SELYE. 1947. L'Université de Montréal; Acta Endocrinologica. Pp. xxxii and 914; illustrated. \$12.80. (Foreign postage \$0.75).

This book provides a comprehensive account of the normal and abnormal structure and functions of the endocrine glands. Following a chapter on general endocrinology, 40 pages are devoted to the occurrence, chemistry and pharmacology of the steroids. Thereafter, each endocrine organ is considered in turn under the headings history, normal morphology, chemistry, pharmacology, experimental physiology and pathology, and diseases causing hypo- and hyper-effects. Finally there is a description of the tumours and cysts of each organ.

The text is both factual and critical. Describing as it does a field of knowledge which is expanding all the time, it would not claim to be complete, nor fully to satisfy the demands of specialists in particular branches. But a very fair account of the present position of our knowledge is presented in readable and interesting form. The bibliographies also make no attempt at completeness. For example, the chapter on the ovary contains only 33 references. A valuable feature is the series of short critical paragraphs indicating the value of each article or monograph referred to: this should be helpful to those who wish to read more widely on any

particular aspect of the subject. The index occupies three columns in each of over 40 pages and is outstandingly well done.

The book is profusely illustrated and contains a unique collection of photographs of patients suffering from endocrine disorders, often taken both before and after treatment. Many of these are very good. One reason for their excellence is the fact that they have been chosen from the collections of many workers, both clinicians and pathologists. The legends also are valuable and well done, but it would have added greatly to the convenience of the reader and to facility of reference from the text if the figures had been serially numbered.

The format of the text, each page being divided into two columns, makes it less easy to read, and the unduly liberal use of dashes is aggravating. In a work of such dimensions it would be ungenerous to stress minor omissions in particular subjects, but the omission of any reference to the pre-pubertal androgenic effects of interstitial-cell tumours of the testis and to Bielschowsky's work on cancer of the thyroid gland might be mentioned in passing.

Without doubt, this book will be of real value to clinicians, pathologists and students alike.

#### The digestive tract in roentgenology

By JACOB BUCKSTEIN. 1948. Philadelphia, London, Montreal: J. B. Lippincott Co. Pp. xvi and 889; 1030 text figs. \$16.

Dr Buckstein some years ago published a very useful book on the roentgenology of the digestive tract. He has now produced a new work which covers much the same ground as the previous volume, but he has enlarged both its scope and its usefulness. The new work covers the whole of the radiological appearances of the alimentary tract and its value is greatly enhanced by the inclusion of a large number of illustrated case histories of the various conditions described. In addition—and this is a most valuable feature—he deals fully with the normal variations in appearance of the digestive tract, and, in the abnormal states, gives a full account of the pathological aspects. This last feature, so often lacking in radiological books, is of great value to pathologist and radiologist alike. It is unusual to find in any one work such an excellent survey of the normal and pathological as revealed by X-ray examination, and, with the high quality of the illustrations, which are all in negative form, this book must become one of the most valuable works of reference in this field. The bibliography is full and up to date, and the vast resources at Dr Buckstein's disposal ensure that the whole range of abnormal conditions has been well covered. The book, though primarily for radiologists, will obviously have a place in the libraries of pathologists, physiologists and all others interested in the digestive tract.

#### Bergey's Manual of determinative bacteriology

By ROBERT S. BREED, E. G. D. MURRAY and A. PARKER HITCHENS. Sixth edition, 1948. London: Baillière, Tindall and Cox. Pp. xvi and 1529. 82s. 6d.

Earlier editions of Bergey's Manual were seldom helpful to a medical bacteriologist seeking to identify a bacterium, because he too often found that the reaction of many of the important tests were not recorded or that differentiation depended on some obscure test or ability to grow on an out-of-the-way medium. In this respect the new edition is little better

than its predecessors and the fault lies with the editors, because they have accepted species that are not fully described. As a result they have had to use vague and indefinite characters in the diagnostic keys. Characters used in the subdivision of *Pseudomonas* include pigment production, action on gelatin and action on milk, yet we find in the key (p. 84) "Green fluorescent pigment not produced or not reported", (p. 85) "Action on gelatin not recorded" and (p. 83) "Action on milk not recorded". The keys would have been much improved if the scheme had been limited to well-described organisms and more of the ill-defined species relegated to the appendices.

Breed has contributed a good survey of the taxonomic proposals of the past, and extracts from the Botanical Code are given to indicate how names should be formed: it is a pity that the new Bacteriological Code was not approved in time to appear in this edition.

The addition of three supplements of ordinal rank to include viruses, rickettsiae and the pleuro-pneumonia group in the scheme of classification of bacteria will arouse interest, but *Virales* at least is likely to have a stormy reception from virus workers. Among the true bacteria *Eberthella* and *Staphylococcus* have disappeared. *Staphylococci* are now in the genus *Micrococcus*; the species recognised and the descriptive details might have been acceptable 15 years ago, now they only show how American thought on this group has lagged behind the work done in this country. *Streptococci* and *corynebacteria* are both inadequately dealt with and the "latest" on the serology of *C. diphtheriae* is Murray's work of 1935. The Kauffmann-White *Salmonella* scheme has been accepted in a half-hearted way and serology has been allowed to influence the shape of *Shigella*. Three sections, *Bacillus*, *Mycobacterium* and *Proteus*, are in line with British thought; for the rest, most workers here will quarrel with the Bergey classification. Taken as a whole the detailed descriptions are better than in the last edition but there is still room for much improvement.

In spite of many shortcomings there is much in this edition of Bergey's Manual that makes it an essential reference book. It is invaluable in tracing synonyms and "The index of names is the most complete list that has appeared in the literature and should always be consulted before new genus or species names are proposed" (p. viii).

#### Endocrinology of neoplastic diseases: a symposium by 18 authors

Edited by GRAY H. TWOMBLY and GEORGE T. PACK. 1947. New York: Geoffrey Cumberlege, Oxford University Press. Pp. vi and 392; 43 text figs. 60s.

Here we are presented with yet another "symposium" by American authors—comprehensive, up-to-date, accurate, and expensively produced. We seem to be diverging a long way from the original purpose of a symposium (a "drinking together"), when, as in this case, a number of papers originally written for publication in *Surgery* in 1944 are brought up to date and published as a monograph. Each of the eighteen authors invited to contribute is an acknowledged expert in his own field and the sum of old and new information provided is a valuable contribution to knowledge.

The subject of the endocrinology of neoplastic disease is considered under two main headings: first, the role of hormones in the genesis of tumours in distant organs such as the breast and testis, and second, the hormonal effects of tumours arising in endocrine organs. The articles of Gardner and Selye are concerned with the first of these subdivisions, in relation respectively to the action of oestrogens and pituitary hormones, while Nathanson discusses the relation of hormones to diseases of the breast.

He inclines to the view that the hormones prepare the tissue for another agent to act as the carcinogen. There is an excellent chapter by Moore presenting all the evidence for and against endocrinological imbalance as a cause of benign hypertrophy and carcinoma of the prostate. The endocrine treatment of prostatic cancer is discussed by Dean, Woodard and Twombly. In the two years which have elapsed since it was written, a good deal more information has been acquired, but the discussion of the principles involved is still valuable.

The rest of the book is mainly concerned with the hormonal effects of tumours arising in endocrine organs. Especially notable is the chapter by Cope on the endocrine aspects of parathyroid enlargement. Much of this ground was covered by Selye in his book "Textbook of endocrinology", reviewed in the present number of this *Journal* (p. 348), a book the wealth of illustration in which adds to its attraction for the general reader.

Of the excellence of presentation in the volume now before us there can be no doubt. Written primarily for the practising surgeon, a vast amount of information is made available in the various articles, each of which is also provided with a comprehensive bibliography. As a work of convenient reference it will fill a need, but in a subject which is growing and changing so rapidly it is doubtful whether many people will be prepared to spend so much money on a book which will be out of date in a very few months or years.

#### Identification of tumors

By N. CHANDLER FOOT. 1948. Philadelphia, London, Montreal: J. B. Lippincott Co. Pp. xxi and 397; 248 text figs. \$6.

The sub-title of this book is "Essential Gross and Microscopic Pathologic Features Systematically Arranged for Easier Identification". It is, in fact, a short treatise on the pathology of the tumours of man, with special emphasis on their morphological aspects. The subject matter is divided into two main sections, nine chapters (96 pages) being concerned with "Neoplasms of general distribution", eleven chapters (257 pages) with "Neoplasms of special systems and organs". By the use of cross references, overlapping is avoided as far as possible. Chap. 21 (8 pages) provides details of technical methods of value in the diagnosis of tumours. Finally there is a "Tabular locator for tentative identification of neoplasms" extending to 25 pages. A very full table of contents and an index complete the volume.

In his preface, Professor Foot states that the book is not intended to be either a synopsis of oncology or a "quiz compend". It is meant to serve as a brief guide to the identity of tumours and to stimulate the observer to further study. "It contains no extended discussions as to theories of origin, no statistical tables, few clinical data and no lists of references". Its contents are "pared down to the essentials necessary for the identification and the diagnosis of a given tumor, with a hint as to prognosis here and there".

Whether a book of this kind will achieve its purpose as thus set forth we have very grave doubts. Its distinguished author would be the first to admit that identification of the less common tumours (not to mention the rarities) can only be achieved by long and intensive study under an acknowledged master. Such a training, we also believe, should start at a fairly early age—not later, shall we say, than 30 or 35. There is no easy way, even by the use of a "tabular locator" such as the one here provided. A really full and comprehensive atlas or series of atlases of tumour histology would be invaluable, but the present volume has no such aspirations. The

publishers describe it (on the jacket) as "truly a quick reference ideally suited for use by student, pathologist, surgeon or practitioner". Well, we just don't believe it.

This is not to say that Dr Foot's book is without value. It contains a great deal of interesting and useful information and there are 248 illustrations, of which no fewer than 150 have already appeared in the author's "Pathology in surgery". All the figures are photomicrographs, and most of them are excellent and well chosen. Naturally enough the approach is individualistic and there are no references. There are, on the other hand, some rather remarkable statements and peculiar usages. The term "metaplasia", for example, is mainly used as synonymous with anaplasia or dedifferentiation, though correctly employed in relation to epidermoid carcinoma of the gallbladder (p. 160). Giant-cell tumours of tendon sheaths are said to be "essentially the same as giant-cell tumors of bone" (p. 57). The normal weight of "a parathyroid" is given as 3 g. (p. 208), neuro-epithelioma is said to be "commoner in the eye and *peripheral nerves* (reviewer's italics) than elsewhere" (p. 285). "Secondary cholesteatomas" of the ear occurring in connection with punctured eardrums are said to be due to the heaping up of keratinised cells from the lining epidermis of the external auditory meatus.

The figures, though serially numbered, are not referred to in the text, a defect which should be remedied in any future edition.

# PROCEEDINGS OF THE PATHOLOGICAL SOCIETY OF GREAT BRITAIN AND IRELAND

9th and 10th July 1948

The seventy-sixth meeting of the Society was held in the University of Leeds on Friday and Saturday, 9th and 10th July 1948.

## Communications and demonstrations

The item marked with an asterisk is abstracted below

- T. D. DAY. The mode of hydration of interstitial connective tissue.
- MARTIN BODIAN. The syndrome of cystic fibrosis of the pancreas.
- LOUISE PEARCE. Hereditary osteopetrosis of the rabbit.
- H. A. SISSONS. Fibrous dysplasia of bone.
- P. A. CLEARKIN and T. H. FLEWETT. Two indigenous cases of granuloma inguinale in Northern Ireland.
- G. R. CAMERON and S. N. DE. Experimental neurogenic pulmonary oedema.
- \*J. E. MORISON. Neoprene latex casts in the study of lung development.
- G. LYMAN DUFF and GARDNER C. McMILLAN. The inhibition of experimental cholesterol arteriosclerosis by alloxan diabetes.
- R. F. OGILVIE. The treatment of alloxan diabetes with anterior pituitary extract.
- W. H. HUGHES. The influence of inflammatory localisation on immunity.
- D. G. EVANS. The effect of incision of infected muscle in experimental gas gangrene.
- A. W. DOWNIE and K. MCCARTHY. Neutralisation tests on the chorio-allantoic membrane of chick embryos with viruses of the variola-vaccinia group.
- S. D. ELEK. The recognition of *Proteus*.
- R. KNOX. A suggested method for rapid "screening" of intestinal organisms.
- G. T. COOK. Urea medium in the bacteriological examination of faeces.
- MARY BARBER. Stability of penicillin-resistance in staphylococci.
- R. R. A. COOMBS and N. H. HOLE. The conglutinating complement-absorption test.
- C. E. LUMSDEN. Experimental "allergic" encephalomyelitis.
- W. GOLDIE, M. GORDON and K. I. JOHNSTONE. Mutation in a single-cell culture of *C. diphtheriae*.
- C. H. BROWNING, K. M. CALVER and H. ADAMSON. The chemotherapy of *T. congolense* infections with phenanthridinium compounds, etc.
- C. L. OAKLEY, G. HARRIET WARRACK and MARION E. WARREN. Antigenic relationship of toxins.
- C. L. OAKLEY, G. HARRIET WARRACK and IRENE BATTY. Antibody production in lymph glands.
- J. ZEISSLER and C. L. OAKLEY. *Cl. welchii* type F and its occurrence in enteritis necroticans.
- J. A. SYKES. Observations upon the effect of boric acid on the flagella of *Proteus* and its possible bearing on flagellar structure.
- W. T. ASTBURY and C. WEIBULL. An X-ray diffraction study of the structure of bacterial flagella.
- A. E. FRANCIS. The use of *B. aerosporus* (Greer) in teaching bacteriology.



- H. PROOM. The mouse intracerebral method of determining the antigenicity of *H. pertussis* vaccines.
- R. D. PASSEY, L. DMOCHOWSKI, W. T. ASTBURY, R. REED and P. JOHNSON. Ultracentrifugation studies of the milk factor.
- I. LOMINSKI, J. B. GIBSON and J. B. KENNIE. Acid-fast bacilli of unknown nature in the sputum and in human lung lesions.
- M. J. STEWART. Giant pacinian corpuscles in relation to the pancreas.
- D. H. COLLINS and W. McI. ROSE. The nature of anaemia in leukaemia.
- R. WILLIAMSON. Cytoplasmic bodies from lymphocytes.
- T. CRAWFORD. Some unusual intracranial tumours.
- A. G. EVERSON PEARSE. The cytochemistry of the gonadatropic hormones.
- R. KNOX and G. T. COOK. Bacteriological examination of faeces: (1) "screening" plate, (2) rapid indole test, (3) rapid urea test.
- K. I. JOHNSTONE. A technique for the micro-manipulation of bacterial cells on the surface of agar, using dark-ground illumination.
- R. F. OGILVIE. The pancreatic islets in alloxan diabetes treated with anterior pituitary extract.
- F. HAWKING, W. L. M. PERRY and J. P. THURSTON. Tissue stages (exocrythrocytic forms) of a mammalian malarial parasite, *Plasmodium cynomolgi*.
- H. A. SISSONS. An epiphyseal abnormality.
- D. J. TREVAN. The occurrence of myocardium in mouse pulmonary vessels.
- C. L. OAKLEY. Congenital occlusion of the common bile duct in a mouse.
- D. H. COLLINS. (1) Lymphosarcoma cells in peripheral blood—"leucosarcoma". (2) Intra-medullary spinal lipoma in a kyphotic spine.
- C. J. POLSON. Nasal pathology illustrated by a collection of specimens.
- D. E. PRICE. A symptomless meningioma invading the sella turcica.

### Abstract

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## NEOPRENE LATEX CASTS IN THE STUDY OF LUNG DEVELOPMENT

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In a large number of fetuses and newborn infants, when neither malformation, birth trauma nor infection is demonstrable, the cause of the failure to establish or maintain aerial respiration may remain obscure, even at post-mortem. The defect may not be primarily in the lungs, but it is essential that these organs should be studied with particular care. Tissue sections, thick or thin, single or in series, fail to give a complete picture of the developing lung. The neoprene latex injection technique used by Duff and More (1944) and Trueta *et al.* (1947) for the blood-vessels of the kidney has been modified, and complete injection and modelling of the air spaces has been attained in both non-expanded and post-natally aerated lungs.

Neoprene latex (B.B. Chemical Co., Leicester) is injected into a bronchus or the trachea at a pressure of 30-35 cm. of water. The material should fill and expand the whole lung uniformly in a few seconds. Mucus, amniotic debris and detached fragments of bronchial epithelium may prevent entry into some areas. This is easy to recognise and, if filling is perfect elsewhere, may not be a serious defect. More serious is the escape of latex into blood vessels and veins, and communications may then appear to ramify widely between the

air spaces. Grossly asphyxiated or macerated foetuses are unsuitable. Only misleading appearances are produced if air is present in the lung at the time of injection. Complete injection into the smallest air spaces is essential. Air, and also amniotic debris, can be displaced through the air passages by saline perfusion through the pulmonary artery. The lung fills with fluid and expands, but collapses when the perfusion is stopped. Methods of exerting uniform pressure on the lung externally so that it will collapse more quickly and completely have been of little value; time, and alternate expansion and collapse of the lung are required. Small quantities of fluid in the air passages may weaken the cast, but do not seem to distort it, and frozen sections should show perfect filling of the air spaces. Corrosion casts are prepared with concentrated hydrochloric acid at 56° C.

The method is inferior to those of Brock (1946) and Liebow *et al.* (1947) for the study of the relationship of the larger bronchi. It is chiefly of value in the study of the ultimate air spaces. For this a binocular microscope with twin objectives is required, and for dissection it has been necessary to fashion knives from wafers broken from the edge of safety razor blades, to modify watch-makers' instruments and to use the finest ophthalmic scissors. The cast is three-dimensional and photomacrography is exceedingly difficult. A 1 in. lens giving a primary magnification of 10 has been most useful with fine-grain miniature films. The lighting of the specimen must be arranged to give modelling and an easy change from binocular microscope to camera is essential.

In formol-fixed material it has been found that the cast gives such support to the air spaces that they can often be separated from one another so that each carries with it its own proper tissues. These tissues can be stained or impregnated before or after dissection and a new clarity of localisation of the different elements is attained. Colour intensity in incident light and the power of the binocular microscope impose limitations here, but the neoprene cast becomes translucent in xylene and many clearing agents and sometimes transmitted light may be useful.

Material is being collected, preserved and photographed to illustrate the growth of the air spaces of the lung from tube-like structures in an abundant mesenchyme to the complex structure of the full-term infant and young child. Elastic-tissue networks can be demonstrated round blood vessels and air spaces and work on other elements is proceeding. The smallest blood vessels will probably require difficult double-injection techniques.

The study of the lung must begin with its development and growth. This neoprene latex technique does not replace other methods but supplements them. Like many techniques it must be considered only as an instrument for a defined purpose.

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## THE CAUSATION OF ACUTE ARTERIAL NECROSIS IN HYPERTENSIVE DISEASE

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(PLATE LXVII)

ONE of the essential features of malignant hypertension is the presence of acute focal necrotic lesions (necrotising arteritis) in the terminal arteries and arterioles of many organs and especially the kidney, where they are closely associated with glomerular necrosis and tubular degeneration. Because these lesions have an "inflammatory" appearance and because they are also found in the kidney in many kinds of primary renal disease complicated by secondary hypertension, malignant hypertension was long believed to be a form of primary nephritis in which the hypertension was a secondary effect of structural renal damage. In recent years, however, careful clinical and histological studies of early cases have brought a growing conviction that malignant hypertension is a form of primary hypertension leading to but not caused by structural renal damage.

When Wilson and Pickering (1937-38) and Goldblatt (1938) reported independently that in animals made hypertensive by Goldblatt's technique typical necrotising arteritis commonly appeared in many organs, it became possible for the first time to study the genesis of the essential lesion of malignant hypertension under controlled experimental conditions. While this opportunity has not been neglected, considerable differences of opinion have been expressed in interpreting the available experimental results.

According to one view, necrotising arteritis in the hypertensive animal is due essentially, if not wholly, to increased intravascular tension and the necrotising arteritis and associated parenchymatous renal damage of malignant hypertension are, by inference, likewise

effects of the hypertension. This view is therefore in complete accord with the modern concept of malignant hypertension. On the other hand, other writers believe that hypertension can cause vascular necrosis only in the presence of a second (renal) factor, variously designated as uræmia and toxic absorption from dead or dying kidney tissue. The implication that serious renal disease precedes the essential lesion of malignant hypertension is difficult to reconcile with the fact that in the early stages of the disease the kidney is often clinically and histologically almost normal. A third view attributes the lesions to renal damage alone and does not concede that increased intravascular tension is concerned.

In this paper we shall attempt to answer three questions relating to the problem, namely: (1) Is hypertension concerned at all? (2) If so, how does it cause the lesion? (3) What additional renal factors, if any, are necessary?

#### *Definition of terms*

The evidence bearing on this problem is mostly derived from two kinds of experiment which must be clearly distinguished.

A *one-kidney experiment* is one in which the circulation to one kidney is impeded by Goldblatt's technique, often with minor modifications, the opposite kidney having been previously excised. The solitary kidney will be known as the "ischemic" kidney. Identical results are obtained in animals with two kidneys, both of which are rendered ischæmic, but to avoid confusion these will be included in the term "one-kidney experiment". This operation commonly causes permanent hypertension.

A *two-kidney experiment* is one in which one kidney is made ischæmic, the opposite (or "intact") kidney, with its vessels, being left undisturbed. In most animals this operation usually causes only a transient hypertension, but in the rat permanent hypertension is common.

The term "vascular lesions" refers to acute focal necrotising arteritis (fibrinoid arterial necrosis). It does not refer to the generalised hypertrophic or degenerative arterial changes, provoked or accelerated by chronic hypertension, which shelter under the omnibus term "arteriosclerosis".

#### **1. Is hypertension concerned at all?**

The conclusion that hypertension is at least partly concerned was first derived from the finding that in one-kidney experiments the kidney remained remarkably free from the vascular lesions which affected many other organs. This has been demonstrated in the rabbit (Wilson and Pickering, 1937-38), the dog (Goldblatt, 1938), the monkey (Goldblatt, 1937) and the rat (Byrom and Dodson, to be published). This finding has generally been interpreted as evidence that increased intravascular tension is concerned in causing the lesions, the clamp on the renal artery acting as a barrier against the raised tension, and this conclusion has been supported by direct measurement of the pressure on the renal side of the clamp (Levy *et al.*, 1938) and by the lack of medial hypertrophy in the renal arteries distal to the clamp.

In two-kidney experiments the evidence, which is based on the rat, is at first sight conflicting. Wilson and Byrom (1939, 1941) reported that vascular lesions occurred in the intact but not in the ischaemic kidney and Friedman *et al.* (1941), who produced hypertension by wrapping one kidney in cellophane, recorded a similar distribution. On the other hand Schroeder and Neumann (1942), using a ligature to constrict one renal artery, found frequent lesions in both kidneys, and Patton *et al.* (1943), with the same technique, also found lesions in the ischaemic kidney, though less commonly than in the intact organ.

TABLE

*Nature and distribution of lesions observed in eight rats (series 1) rendered hypertensive by constriction of the left renal artery with a cotton thread ligature*

Rat no.	Systolic blood pressure (mm. Hg.)*			Lesions in kidney				Arterial necrosis (recent or healed) in other organs
	Range	Mean	Duration (weeks)	Side	Medial hypertension	Arterial necrosis	Parenchymatous changes	
30 B	128-175	152	13	Left Right	— +	— +	Atrophy and infarction Glomerular and tubular lesions	None
30 LC	130-175	143	10	Left Right	+ +	— +	Atrophy + None	+ Heart
30 BC	123-190	160	14	Left Right	— +	— +	Atrophy and infarction Tubular and glomerular lesions	+ + Heart, brain, mesentery
32 BO	130-145	138	3	Left Right	— —	— + +	Atrophy + Glomerular necrosis	+ + Heart
32 LC	133-195	168	17	Left Right	— +	— +	Very slight atrophy Glomerular and tubular lesions	+ + Mesentery
32 BC	140-170	153	3	Left Right	— —	— —	Medial atrophy None	+ + Heart, brain
32 RS	122-190	153	14	Left Right	— +	— —	Slight atrophy None	None
32 LS	138-190	159	7	Left Right	— +	— +	Moderate atrophy Glomerular and tubular lesions	+ + Heart, mesentery, spleen

\* Systolic blood pressure was measured under ether anaesthesia, using an optical method (Byrom, 1947). In normal rats the mean value is  $115 \pm 12$  mm. Hg.

Since Byrom and Wilson used a silver clip to compress the artery, except in a few preliminary experiments, we have re-examined this question in a short series of 12 two-kidney experiments on young adult albino rats (series 1), in

which the left renal artery was constricted by a cotton thread ligature to an estimated internal diameter of 0.25 to 0.35 mm. Hypertension occurred in 8 rats and, of these, 6 showed vascular lesions in the intact kidney and 6 in other organs (table). No vascular lesions were found in the ischæmic kidney. In our hands, therefore, ligatures are as effective as clamps in protecting the kidney from vascular damage.

The tabulated data of Schroeder and Neumann suggest an explanation for their divergent findings. In their series of 34 rats examined histologically, arteriolar necrosis in the ischæmic kidney is mentioned in 12 instances. In each of these 12, and in only 4 of the remaining 22, medial hypertrophy of the arteries of the ischæmic kidney is also recorded. Whatever may be the cause of necrotising arteritis, there is no doubt that medial hypertrophy is a physiological adjustment to sustained hypertension and that, therefore, in the rats that displayed vascular lesions in the ischæmic kidney the ligature had provided an incomplete or inconstant barrier to the increased tension.

The available evidence from these several sources, therefore, all points to the conclusion that an effective barrier on the renal artery protects the kidney from vascular damage, which suggests very strongly that increased intravascular tension plays a direct part in causing vascular lesions. It does not, of course, follow that it is the only factor concerned.

## 2. How does increased intravascular tension cause arterial necrosis ?

The sequence may be most readily explained by assuming that an intravascular tension bordering on the limits of tolerance leads directly to sudden local overstretching of the vascular wall, the overstretched muscle undergoing necrosis and lysis. Plasma and blood may then seep into the weakened zone to be converted into a fibrinoid plug by thromboplastin liberated from the dead muscle, and an inflammatory infiltration would be expected to follow in the adventitia as a first step towards repair. Such a sequence would be in harmony with the abrupt onset of the lesion,\* its tendency to follow sharp rises in the blood pressure curve (Wilson and Pickering, 1937-38; Wilson and Byrom, 1939; Byrom and Dodson, unpublished), its focal distribution, its necrotic nature and general histological characters, and its tendency to heal rapidly by fibrosis if the hypertension is relieved (Byrom and Dodson). The hypothesis can, however, be submitted to more direct test, for, if it is true, it should be possible to produce typical arterial necrosis in a normal animal by brief artificial overdilation of the arterial tree. This possibility has been tested as follows :—

In a series of 23 normal young adult rats (*series 2*), the left common carotid artery was exposed under ether anaesthesia, dissected clear of the vagus and

\* Lesions have been observed as early as three days after constricting the renal artery (Wilson and Byrom, 1939).

ligatured at its upper end. Two additional ligatures were placed, ready for tying, on the cardiac side of the first. A short needle, bore 0.6 mm., bent to an angle of 135 degrees and attached through a 3-way tap to a 2-c.c. Record syringe, was inserted into the carotid, directed towards the heart and tied in position with the middle ligature. Warm Ringer's solution was then drawn into the syringe and injected into the artery as forcibly as possible. This procedure was repeated from ten to fifteen times, after which the carotid was ligatured and the needle withdrawn. In ten rats a length of thread was looped round the left renal artery and traction was applied during each injection to protect the kidney against the increased tension.

In a control experiment a wide bore needle connected with a mercury U-tube manometer of 1.0 mm. bore, was inserted into the lower abdominal aorta, the rat having been first heparinised. Each intra-carotid injection was observed to cause a sharp rise of pressure in the manometer followed by an almost equally rapid fall to the previous level within two to three seconds. The increase in mean pressure observed was from 80 to 90 mm. Hg., but this figure must be accepted with reserve, because a mercury manometer is ill adapted to measuring such rapid changes. There was, however, no doubt that the rises were transient and that, as the experiment proceeded, the resting pressure tended to fall markedly. In several animals the kidney was observed throughout the experiment. During each injection the cortex blanched momentarily as the wave of saline swept through. At first the normal colour was immediately regained but after several injections residual pallor was sometimes observed, usually limited to small irregular areas which slowly changed in shape and size. These changes were evidently due to transient spasm of the underlying arteries in response to the mechanical insult. The injected fluid evidently escaped from the blood stream very promptly, for the pancreas and mesentery became grossly oedematous after several injections. Apart from temporary apnoea in some instances, the rats recovered quickly and completely from the experiment and seemed quite well when killed three days later.

At post-mortem examination the kidney, except where protected by traction on the artery, displayed either pinhead yellow flecks or larger areas of subcapsular pallor, and in a few rats similar changes were seen on the surface of the heart. Microscopical examination of the kidneys showed typical acute necrotising arteritis in ten of the 23 rats (figs. 1-4). The lesions were focal and usually limited to one or two vessels in a complete section. The vessels involved were the arcuate and interlobular arteries and the afferent glomerular arterioles, and the essential lesion was necrosis of the muscle, often involving only a segment of the circumference. The affected fibres were autolysed and obscured by an exudate which sometimes extended into and beyond the adventitia, and contained irregular, brightly eosinophilic zones of fibrinoid material. Red corpuscles were often present in the lysed media. In the adjacent intact muscle some nuclei were vesicular and pale, some were pyknotic, and others displayed karyokinetic figures. The intimal endothelium was sometimes displaced inwards by a watery or fibrinoid exudate, the elastica being sometimes stretched and fragmented. In the adventitia a conspicuous cuff of inflammatory cells, including neutrophil and eosinophil leucocytes, lymphocytes, monocytes and spindle fibroblasts, was usually present. In occasional arteries the lesion consisted of



simple coagulative necrosis of the media. As in human and experimental hypertension, the histological picture varied in different lesions. Hypertensive glomerular lesions were not observed. In a few instances where the lumen of the affected artery was narrowed by intimal exudate the adjacent parenchyma was infarcted, but as a rule parenchymatous changes were limited to moderate tubular degeneration.

Traction on the renal artery was found to have protected the kidney against arterial lesions. In one such kidney, however, a small anæmic infarct was observed, and in another a primary branch of the renal artery outside the kidney showed a small area of hyaline medial necrosis, possibly a direct result of traction. No vascular lesions were observed in other organs, but in one rat extensive acute necrosis of the mucosa of the stomach, with œdema of the submucosa, was found. Identical gastric lesions have been described in the rat after large doses of vasopressin (Dodds *et al.*, 1934; Byrom, 1937).

This experiment shows that sudden brief rises in intra-arterial tension can directly cause typical fibrinoid arterial necrosis in a normal animal and that the smaller arteries of the kidney are selectively vulnerable. It suggests that the relationship between increased pressure and vascular necrosis in experimental and human hypertension may be equally simple and direct, the arterial muscle giving way suddenly and locally under the strain and provoking a vigorous attempt at repair.

It is evident from the present experiment that while occasional renal arteries subjected to mechanical strain undergo necrosis, many more react by a spasmodic contraction intense enough to blanch the adjacent cortex. The lack of necrosis of the renal parenchyma, which is very sensitive to sustained ischæmia, indicates that these contractions are very transient and are probably not, at least in the present circumstances, an essential link in the chain leading to arterial necrosis. The occurrence of spasm is, however, interesting in view of the widespread suspicion that some of the acute symptoms in malignant hypertension, and especially those associated with encephalopathy, may be caused by transient vasospasm.

### 3. Are other factors concerned?

In his original paper on this question, based on one-kidney experiments, Goldblatt (1938) expressed the opinion that vascular lesions appeared in the hypertensive animal only when the artery was clamped tightly enough to cause renal insufficiency. Fasciolo and Cramer (1938) reported that the blood urea is not necessarily raised in animals displaying vascular lesions, but Goldblatt points out that the blood urea may be normal in "uræmia," and in one-kidney experiments it is admittedly difficult, without frequent

## HYPERTENSIVE ARTERIAL NECROSIS

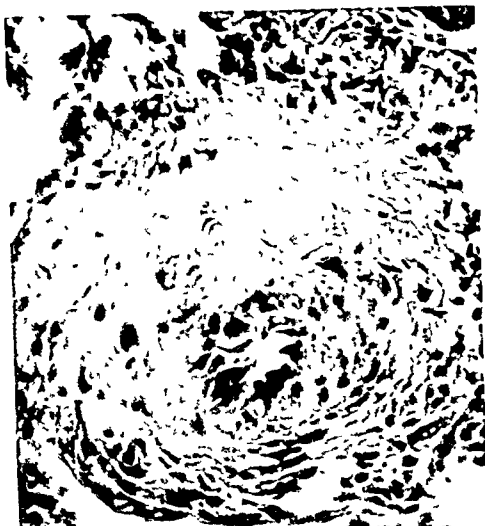


FIG. 1.—Focal fibrinoid necrosis of a small renal artery. The media of about one-half of the circumference of the vessel has lost its structure and merges into a broad zone of fibrinoid exudate, in the periphery of which are numerous inflammatory cells. Hæmatoxylin and eosin.  $\times 150$ .

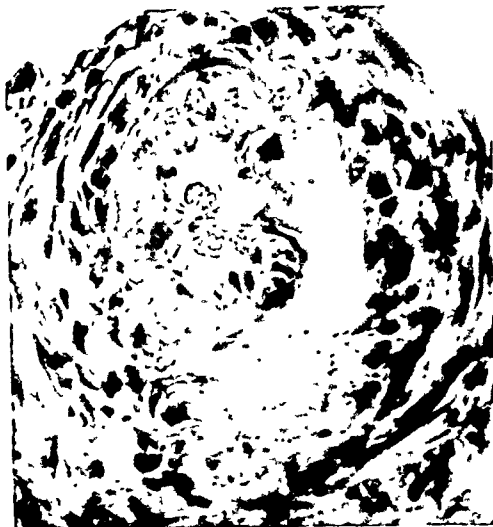


FIG. 2.—Focal fibrinoid necrosis of a small renal artery, showing focal replacement of the medial muscle by fibrinoid material and a perivascular cuff of inflammatory cells and fibroblasts. Two nuclei in the vessel wall are greatly enlarged and display karyokinetic figures. Hæmatoxylin and eosin.  $\times 200$ .

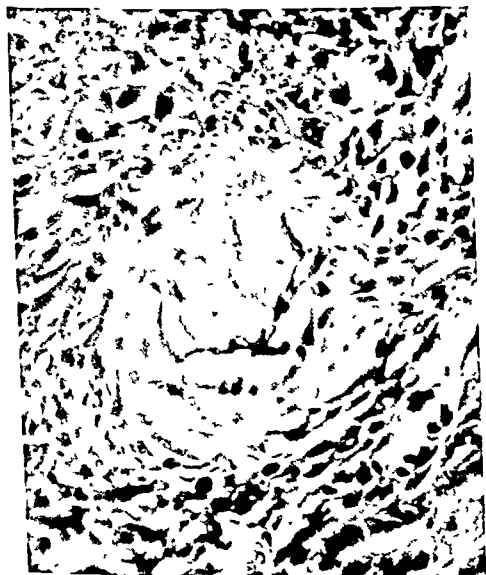


FIG. 3.—Sub-intimal exudate in a small renal artery, showing a localised area of watery exudate between the endothelium and the internal elastic lamina. The adjacent medial muscle is oedematous and an intense inflammatory infiltration is visible in the adventitia. Hæmatoxylin and eosin.  $\times 210$ .



FIG. 4.—Simple coagulative medial necrosis of a renal arteriole. The vessel is dilated and the media is structureless and devoid of nuclei. The intima and adventitia are normal. Hæmatoxylin and eosin.  $\times 180$ .



measurements of renal efficiency, to deny that renal failure may not occur at some stage of the experiment.

The subsequent demonstration by Wilson and Byrom (1939) of vascular lesions in rats with a second intact kidney seemed to them strong evidence that uræmia was not an essential factor. Goldblatt, however, has not accepted this conclusion and in his latest paper (1947) he suggests that it was based on "the erroneous assumption that the contralateral kidney was normal" and states that this, the intact kidney, must have been affected by spontaneous pyelonephritis or hydronephrosis which, he asserts, are common in apparently normal rats. He further suggests that some of the renal lesions attributed by Wilson and Byrom to hypertension were in fact due to such spontaneous renal disease.

Implicit in Goldblatt's objection is the assumption that in all two-kidney experiments in which necrotising arteritis is found in the intact kidney or other organs, the intact kidney must have been the seat of pre-existing spontaneous pyelonephritis or hydronephrosis. This assumption is not valid, for the following reasons.

In the first place the incidence of vascular lesions in two-kidney experiments is very high. In series 1 of the present experiment, seven out of twelve rats developed arterial necrosis. In Wilson and Byrom's much larger series (1941) exact figures could not be given, for reasons stated by the authors, but an incidence of 50 per cent. would be a conservative estimate, in the opinion of one of the authors (F. B. B.). Schroeder and Neumann also stated that lesions appeared "with great regularity" in two-kidney experiments. The animals used in all these experiments were young adults, and it therefore becomes necessary to postulate that something like 50 per cent. of young rats are afflicted with spontaneous renal disease. Although Wilens and Sproul (1938) have reported a fairly high incidence (23.8 per cent.) of destructive renal disease, due perhaps partly to pyelonephritis, in aged rats, they stated that such changes were uncommon in young rats. This has been our own experience and we are not aware of any evidence to the contrary. Secondly, in the early stages of two-kidney hypertension, the acute arterial and glomerular necroses in the intact kidney, which bear no resemblance to the lesions of uncomplicated pyelonephritis, are focal, leaving wide areas of unaffected tissue, which should show histological evidence of the pyelonephritis or hydronephrosis postulated by Goldblatt. No such evidence was observed either by Wilson and Byrom or in the present series. Thirdly, in the later stages of two-kidney experiments, the intact kidney may present a grossly abnormal appearance. Wilson and Byrom were satisfied that this was simply the end result of cumulative acute vascular damage. If it be assumed that they were in error and that this damage was due to spontaneous renal disease, it still remains to account for the absence of histological evidence of this spontaneous disease in the opposite (ischæmic) kidney.

For these reasons we conclude that renal insufficiency, in the ordinary sense of the term, cannot convincingly be incriminated as necessary for the production of arterial necrosis in two-kidney experiments. This is not to say, however, that uræmia, when present, may not facilitate the development of vascular lesions.

More recently a dual hypothesis of a different kind has been suggested by the work of Winternitz and his associates,\* who compared the effect in the dog of bilateral nephrectomy, complete occlusion of both renal arteries and ligation of both ureters. In the first group death occurred in seven days and few lesions were observed apart from scattered hæmorrhages and slight œdema. In the second group death occurred in 3-4 days, and vascular lesions resembling those of malignant hypertension in appearance and distribution were found. Ureteric ligation gave a similar picture except that pronounced necrotic lesions were found in the glomeruli.

In series 2 of the present study we have repeated these experiments. Four groups of rats were used.

Group "A" consisted of 7 rats subjected to bilateral nephrectomy. The average survival time was 76 hours. The heart and pancreas were examined microscopically. In two rats minute areas of necrosis were observed in the myocardium, the dead fibres being partly lysed and replaced by erythrocytes and inflammatory cells. No vascular lesions were discovered.

Group "B" consisted of 6 rats in which both ureters were ligatured. The average survival time was 71 hours. In 2 rats typical necrotising arteritis was found in the pancreas and in the peri-pelvic connective tissue of the kidneys. The lesions were very scanty and involved the terminal arteries. In both animals and in two other rats, focal cardiac lesions similar to those occurring in Group "A", were found. These were more evident in the right ventricle and they differed from the cardiac necroses found in rats with experimental hypertension in that there was no evidence of necrotising arteritis of the adjacent coronary vessels. The kidneys appeared normal apart from slight dilatation of Bowman's capsules, the convoluted tubules and the renal pelvis.

Group "C" was composed of 7 rats in which both renal arteries were completely occluded by ligation. The average survival period was 48 hours. No cardiac lesions were found in this group. Typical necrotising arteritis was found in the pancreas of 2 rats. The kidneys were completely infarcted.

Group "D" comprised 2 rats in which the left renal artery was occluded by ligation. The rats were killed 3 days later but no lesions apart from infarction of the kidney were found.

These results confirm the American report that bilateral ligation of the renal arteries causes earlier death than nephrectomy and that necrotising arteritis may follow ligation of either both renal arteries or both ureters. In harmony with these findings is the observation by Winternitz and his associates and by Leiter and Eichelberger (1943) that injection of kidney and other tissue extracts causes vascular lesions in animals only if renal insufficiency is present. Although many writers have reported rises in blood pressure after ligation of

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\* Owing to the war we were unable to obtain Winternitz's original papers. Our information is derived from Braun-Menéndez *et al.*, 1940.

the renal arteries or ureters, the increase is too small to account satisfactorily for the vascular lesions observed. In the present series we were unable to obtain blood pressure readings except within the 24 hours of the various operations, and the readings were all within the normal range.

It therefore appears that necrotising arteritis may be caused experimentally, in the absence of gross hypertension, by a combination of total renal insufficiency and total necrosis of the kidney. This combination is never present in either experimental or human hypertension and it is well known that necrotising arteritis occurs in many conditions, *e.g.* peri-arteritis nodosa, in which hypertension is absent. We have already seen that in the hypertensive animal increased vascular tension is concerned in causing lesions and that uræmia is not necessary. Is it possible that absorption of toxic material from dead and dying tissue in the ischæmic kidney is a necessary adjunct to increased vascular tension in causing lesions?

In the case of one-kidney experiments it is generally agreed that the solitary kidney remains substantially or entirely free from structural damage in the dog (Goldblatt, 1938), the rabbit (Wilson and Pickering), and the rat (Byrom and Dodson). Renal necrosis cannot therefore be an essential factor in one-kidney experiments. In two-kidney experiments, on the other hand, the ischæmic kidney is usually atrophied, sometimes to an extreme degree, and not uncommonly infarcted as well. However, in a considerable number of rats in Wilson and Byrom's series in which vascular lesions were found, the ischæmic kidney showed only slight or negligible atrophy and, in many more, evidence of infarction was absent. The functional efficiency of the ischæmic kidney was, in fact, such as to permit successful excision of the opposite kidney in a considerable number of the rats. Rat 32 L.C. in series 1 of the present research is typical of this kind of experiment. The animal was a young male weighing 160 g. which developed a steady hypertension of 160-180 mm. Hg. after constriction of one renal artery. Sixteen weeks later the rat suddenly collapsed and died from intraperitoneal hæmorrhage and gross necrotic lesions were found in the mesenteric arteries. Both kidneys appeared macroscopically normal, the left (ischæmic) organ weighing 0.86 g., the right 0.93 g. Assuming that atrophy of the former was balanced by compensatory hypertrophy of the latter the degree of atrophy of the ischæmic kidney was less than 4 per cent. by weight. Histologically this kidney appeared normal, while the right kidney showed scattered chronic vascular lesions. It may therefore be said that while evidence of necrosis or gross atrophy of the ischæmic kidney or of both is common in two-kidney experiments, there are important exceptions in which this kidney is almost normal and in which extensive vascular lesions are nevertheless present elsewhere. Any hypothesis as to the genesis of vascular lesions must cover these exceptions. Braun-Menéndez *et al.*, in an ingenious attempt

to reconcile the available evidence, suggest that arterial necrosis may, in different circumstances, arise from a combination of any two of the three factors, hypertension, uræmia and toxic absorption from dead renal tissue. Even this elastic hypothesis does not explain the appearance of lesions in experiments in which clear evidence of either renal failure or infarction is lacking.

## DISCUSSION

Our interpretation of the available evidence is that it provides strong grounds for accepting the simple mechanical explanation of acute hypertensive vascular lesions, a view which is entirely in harmony with the modern clinical concept of malignant hypertension, and implies that the difference between benign and malignant hypertension is simply one of degree. The possibility of a second renal factor cannot, however, be absolutely excluded, but as we have seen, to satisfy the evidence, such a factor must be one which can, on occasion, be derived from a degree of renal ischæmia too small to cause more than a very slight simple atrophy of one kidney. When qualified in this way the dual hypothesis can be largely reconciled with the clinical evidence for the following reasons. The clinical conclusion that malignant hypertension is a form of essential hypertension rests on the observations that in the early stages of the disease there is no clinical evidence of renal disfunction and that, where it has been possible to examine the kidney histologically, no obvious changes apart from occasional acute vascular and glomerular necroses may be found. But when renal function is examined by the more sensitive clearance techniques developed by Homer Smith and his associates (Goldring *et al.*, 1941), malignant hypertension is found to be accompanied by early abnormalities in the shape of reduced renal blood flow and diminution in the total mass of functional tubular tissue. There is, therefore, in early malignant hypertension at least as much evidence of renal ischæmia as is found in some animals with vascular lesions.

It might therefore be argued that this renal ischæmia contributes a "toxic" factor which is an indispensable adjunct to hypertension in causing vascular necrosis. If so, it remains to account for the absence of arterial necrosis in benign hypertension, where the renal blood flow and tubular excretory mass are also reduced, and it becomes necessary to postulate that the renal ischæmia is greater or develops more rapidly in malignant hypertension; in other words, that there is no qualitative difference between the two forms of essential hypertension. For these reasons we suggest that there is no fundamental conflict between the clinical and experimental evidence regarding the nature of malignant hypertension.

The case for an essential "toxic" factor of renal origin nevertheless lacks direct support and it seems sufficient to accept hypertensive

arterial necrosis as an expression of simple vascular strain. It is significant that the lesions are confined to the terminal arteries and arterioles of the greater circulation. These vessels interpose between the heart and the capillaries a powerful dynamic barrier and are normally exposed to great stresses which are progressively increased in hypertension. In the malignant form of essential hypertension the strain is apparently greater, or is applied more rapidly, than in the benign form and causes a succession of minute catastrophes in these vessels. As Wilson and Byrom (1941) have pointed out, the participation of the renal vessels in this destructive process is of particular significance, since it reinforces the original hypertension and initiates a vicious circle.

Lastly we have seen that, while there are good grounds for rejecting both uræmia and frank renal necrosis as essential factors in causing arterial lesions, there is some experimental evidence that either may, when present, aggravate the production of lesions in hypertensive animals. It is therefore quite possible that these factors may play a secondary part in precipitating a "malignant" termination in chronic renal hypertension and may accelerate the later stages of true malignant hypertension.

#### SUMMARY

1. The available evidence indicates that increased intravascular tension is directly concerned in causing acute arterial necrosis in the hypertensive animal and, by inference, in human hypertensive disease.

2. Neither uræmia nor "toxic" absorption from injured renal tissue is a constant or necessary factor in the process, but it is possible that either may, when present, facilitate the production of lesions.

3. The lesion probably represents a vigorous attempt to repair a threatened breach due to local death and lysis of overstretched medial muscle fibres in the terminal arteries and arterioles.

4. In harmony with the above conclusions it is shown that brief artificial overdistension of the arterial tree in the normal rat causes typical focal arterial necrosis, the smaller arteries of the kidney being selectively vulnerable. The same procedure causes more widespread but transient focal spasm of the small renal arteries.

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# MUTATION IN A SINGLE-CELL CULTURE OF *CORYNEBACTERIUM DIPHTHERIÆ*

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(PLATE LXVIII)

McLEOD (1943), in a comprehensive review of the three types of *Corynebacterium diphtheriæ*, defined their distinguishing criteria and discussed type stability. Though temporary variations may occur in unfavourable cultural conditions, there is much evidence of the general stability of the three types. Isolated cases have been reported, however, of spontaneous mutation under normal cultural conditions. Robinson (1934) described a change of type by the formation of papillæ on colonies of an old *intermedius* strain. Subcultures from these papillæ gave colonies of *gravis* appearance, though they fermented starch only after several months' training in starch-containing media. Mair (1936) described the development of rough variants arising as papillæ on colonies of "barred starch-fermenting" strains. Carter (1946), in the routine examination of 1000 positive throat swabs over a period of 9 months, discovered 14 strains which showed a similar change. These also were initially starch fermenters.

Two strains of *C. diphtheriæ* similar to those described by Carter were recently isolated at Northallerton. When first cultured on unheated blood-tellurite medium (Johnstone and Zinnemann, 1943) they had the typical colony appearance of *intermedius* strains, but also fermented starch. After three days' incubation all colonies showed from 1 to 6 papillæ, subcultures from which gave typical *gravis* colonies. The original strains and their mutants will be referred to as "*intermedius*" and "*gravis*" respectively. From one case (Robins) the strain was isolated in the "*intermedius*" phase on 25 occasions, showing mutation in culture only; on no occasion was a "*gravis*" colony isolated on primary culture. From the second case (Lavin) the strain was twice isolated in the "*intermedius*" phase, but on 16 subsequent occasions the strain recovered invariably appeared to be the "*gravis*" mutant. No epidemiological relationship was found between the cases from which these strains were isolated, there being an interval of two months between them. They were of moderate severity, with no complications, and the patients, aged 7 and 21 years respectively,

who had been immunised, were both nursed in cubicles. The strains were studied after culture from single cells, one (Robins) being finally cultured from an organism obtained after four successive single-cell isolations, with several intervening subcultures, each from a single "intermedius" colony showing no evidence of papillæ.

### *Technique of single-cell culture*

A slab of clear nutrient agar 0.083 in. thick was cast in a sterile rectangular brass mould placed face downwards on a sterile glass slide, the latter being removed by a sliding motion when the agar had set. The agar was then cut into smaller blocks 0.25 × 0.75 in. with a sterile knife, and each was placed on a sterile slide, the surface cast in contact with the glass being uppermost. For *C. diphtheriæ* it was necessary to enrich the medium by spreading one drop of sterile ox serum on the surface of each block and incubating at 37° C. in a Petri dish until visible fluid had disappeared. The transparency of the medium remained unimpaired.

A faintly turbid suspension of the strain from an 18 hours' growth on heated blood agar was made in nutrient broth, and a minute loopful inoculated on to one end of an agar block and allowed to dry. The slide carrying the agar block was placed on the mechanical stage of the observing microscope (Johnstone, 1943), the agar being protected from air contamination by a rectangular Perspex cell with an opening at one end for the introduction of the micro-needle, and having a sliding cover interlocking with dust-excluding collars on the objectives and allowing of free movement of the stage.

The microscope lamp of Barnard and Welch (1936), with a 100-watt bulb and a  $\frac{1}{2}$  in. glass rod, was employed. A Holooscopic immersion condenser (Watson), used dry and with the top lens replaced by the plano-convex eye lens of a no. 1 Leitz ocular, had sufficient working distance to focus through the slide and agar block. Cell isolations were carried out under a  $\frac{1}{2}$  in. parachromatic objective (Watson), with a diaphragm 0.147 in. in diameter behind the back component to reduce the aperture sufficiently for dark-ground illumination. This was obtained simply by swinging into the optical axis a patch stop (0.5 in. diameter) in the substage carrier ring and opening the iris fully. A compensating ocular ( $\times 18$ ) was used throughout.

Glass micro-needles, functioning as the metal needle of Koblmüller and Vierthaler (1933), were used to remove selected organisms from the edge of the inoculated area. They were made by drawing out thin glass rod (0.04 in. diameter) by hand in a micro-burner, the tips being bent downwards towards the agar surface. A steel needle-holder carried the needle horizontally and was clamped rigidly to one aperture of the rotating nosepiece of a second or manipulating microscope by means of a brass nosepiece attachment with the standard objective thread. The needle tip was centred in the field by sliding the manipulating microscope on the smooth bench surface, both microscope stages being horizontal. The minute image of the light source at the upper surface of the agar was a valuable guide when adjusting the micro-needle. Vertical motion of the needle was effected by the focussing adjustments of the manipulating microscope; horizontal motion in both planes by the mechanical stage of the observing microscope, which moved the agar surface relative to the needle tip. The needle could readily be swung out of the optical axis by rotation of the nosepiece of the manipulating microscope.

A single organism was drawn away from the periphery of the inoculated area in the cone of water which surrounds the needle tip when in contact with the agar surface (Koblmüller and Vierthaler), the organism following in the wake of the needle and being under constant observation. After removal for

several millimetres the needle was raised, leaving the solitary organism on the agar surface.

The needle-holder was then exchanged for a platinum needle which could be electrically heated (Johnstone) and was carried by a metal mount interchangeable in the nosepiece attachment. The marking of the position of individual organisms, the incubation of the agar block and the method of subculture were all as previously described (Johnstone), except that, owing to the small size of the colonies of *C. diphtheriæ* after 8-12 hours' growth, inoculation was made directly with the tip of the platinum needle to the surface of heated blood agar instead of washing off the organisms from the needle with a loopful of broth. The micro-needles, Perspex cell, agar mould and slides were sterilised by exposure for 6 hours to the vapour of 40 per cent. formaldehyde solution in a desiccator which had a small electric bulb incorporated to prevent condensation of water vapour.

The advantages of this modified technique are (1) complete isolation of the developing colonies and consequently fewer microscopical examinations during incubation to exclude overgrowth by neighbouring organisms; (2) the area originally inoculated can, in the case of rapidly growing organisms, be removed after severing the agar block with a sterile knife through a line of pits made by the platinum needle, thus eliminating the possibility of overgrowth on to the isolated cells; (3) more rapid manipulation and the advantage of direct or dark-ground illumination at will, enabling numerous isolations to be made without undue eye-strain.

#### *Growth on various solid media*

The strains were inoculated on to the following media, of which the tellurite media were prepared with a low-temperature extract broth (Anderson *et al.*, 1931), the others with autoclaved Wright's broth.

(1) *Blood-tellurite media.* On *saponin-laked blood-tellurite agar* after 24 hours' incubation the isolated colonies, about 0.5 mm. in diameter, were circular with entire edge and had a smooth surface with a dark grey centre and translucent periphery, *i.e.* they were typical *intermedius* colonies. After 48 hours' incubation they had reached 1 mm. in diameter and still had a dark grey centre and translucent periphery, but many now presented 1-6 papillæ. These were from 0.1 to 0.3 mm. in diameter, paler grey than the mother colony and with an almost smooth shiny surface.

With continued incubation the papillæ assumed a dark leaden colour, spread and coalesced so that by the fourth day some of the original "*intermedius*" colonies, particularly where well isolated, were almost completely overgrown and appeared as "*gravis*" colonies. Where the colonies were more congested, though still discrete, the papillæ remained small and did not coalesce, and where the growth was confluent many colonies had no papillæ. If, however, the persisting "*intermedius*" colonies were subcultured to the same medium, papillæ developed as before. After 7-14 days' incubation some isolated colonies appeared as typical large *gravis* colonies up to 5 mm. in diameter. Suspensions of such whole colonies in broth, inoculated to tellurite medium, yielded almost pure "*gravis*" growths with less than 1 per cent. of "*intermedius*" colonies.

On *heated blood-tellurite agar* papillæ also developed readily (figs. 1-3). The papillæ at any stage of their development gave pure "*gravis*" growths on subculture.

(2) *Nutrient agar*. The strains grew readily but showed only slight mutation, scanty papillæ being seen usually after 5 days' incubation. On occasional batches of nutrient agar, mutation occurred more readily.

(3) *Serum agar*. Addition of 10 per cent. of horse serum to the agar, though producing larger colonies, did not stimulate the development of mutant papillæ. On nutrient agar enriched with 50 per cent. of ox serum and 0.0125 per cent. of cystine (Clauberg *et al.*, 1936), the colonies showed a great increase in size and were indistinguishable from those of the "*gravis*" phase, though on subculture to tellurite medium they still showed typical *intermedius* appearance and size. Colonies of a normal *intermedius* strain on the serum-cystine agar were very much smaller. The abnormal size of colony in the "*intermedius*" phase was due almost entirely to the serum, omission of cystine merely resulting in a slight diminution of colony size in both phases. No mutation was observed on these media.

(4) *Blood agar*. (a) On *unheated blood agar*, papillæ appeared early, being well marked after only two days' incubation and often overgrowing the mother colony by the third day. (b) On *heated blood agar* papillæ did not develop so readily, not appearing before the third or fourth day and then only on well-isolated colonies. After 24 hours' incubation the "*intermedius*" growth showed a greenish appearance and this characteristic feature was enhanced by enrichment of the medium with unheated rabbit serum (Gordon and Higginbottom, 1942). The opaque white papillæ which developed later stood out in sharp contrast (fig. 4).

In view of the absence of mutation on serum agar and the striking appearance on blood agar, the effect of the addition of unheated washed horse-blood corpuscles to agar was investigated. It was found that, as compared with nutrient agar, mutation was stimulated in spite of a smaller colony size, papillæ being visible to the naked eye after two days' incubation. If low-temperature extract broth (Anderson *et al.*) were used instead of Wright's broth in the preparation of blood agar, the formation of papillæ was also greatly stimulated.

#### *Appearance of colonies of the mutant*

The mutant was also cultured from a single cell of a growth obtained after successive subcultures from a "*gravis*" papilla. On unheated blood-tellurite medium the colonies were opaque, without the translucent periphery of the "*intermedius*" type and had a matt surface. They were paler grey than those of the mother strain, twice the diameter after 24 hours' incubation and nearly thrice the diameter after 48 hours. On heated blood-tellurite medium the daisy head



### PLATE LXVIII

FIG. 1.—Colonies of the strain Robins on heated blood-tellurite agar after 24 hours' incubation.  $\times 7.5$ .

FIG. 2.—The same colonies after 48 hours' incubation, showing multiple papillae.  $\times 7.5$ .

FIG. 3.—Two of the colonies from fig. 2 enlarged to  $\times 18.5$ .

FIG. 4.—One colony of strain Robins on serum heated-blood agar, with opaque white mutant papillae.  $\times 17.5$ .

FIG. 5.—Colonies of the mutant from strain Robins on heated-blood tellurite agar after 24 hours' incubation.  $\times 7.5$ .

FIG. 6.—The same colonies after 48 hours' incubation, showing the typical daisy head appearance.  $\times 7.5$ .

FIG. 7.—The strain Lavin in the "*intermedius*" phase, from a culture on heated-blood agar, showing the characteristic barring. Methylene blue.  $\times 1300$ .

FIG. 8.—The strain Lavin in the "*gravis*" phase on the same medium, showing more uniform staining. Methylene blue.  $\times 1300$ .

MUTATION OF *C. DIPHTHERIA*

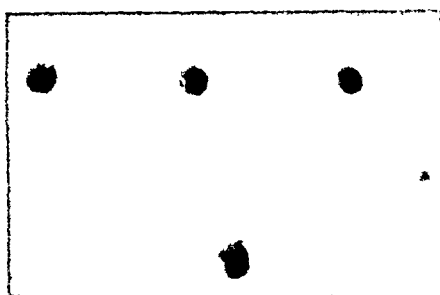


FIG. 1.

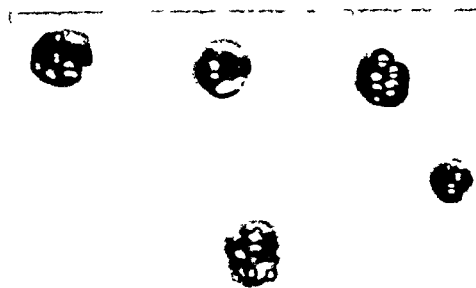


FIG. 2.

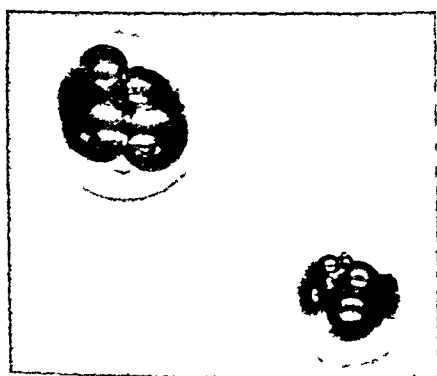


FIG. 3.



FIG. 4.

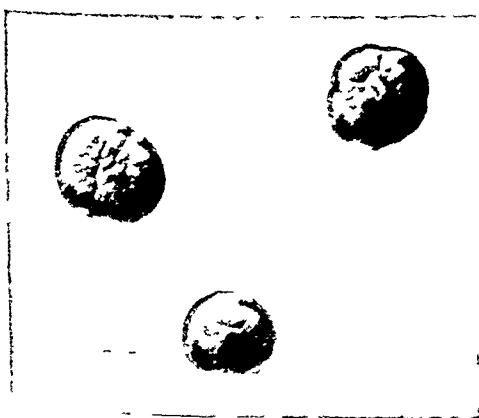
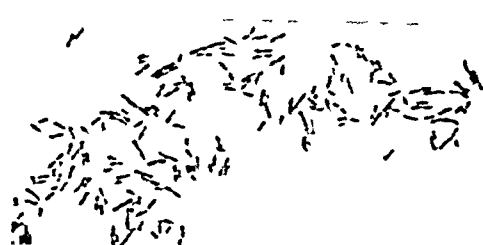
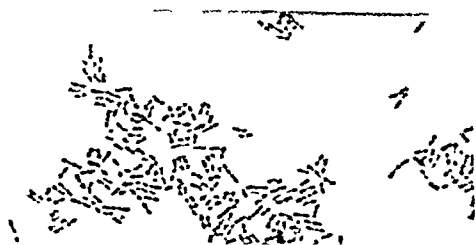


FIG. 5.



FIG. 6.







colony, typical of *gravis* strains, was well marked (figs. 5 and 6). These mutant colonies were of a much firmer consistency than the soft, easily pulped "*intermedius*" colonies and could be pushed along the surface of the medium with a loop. On unheated blood-agar colonies of the mutant and of the "*intermedius*" strain showed no evidence of hæmolytic.

#### *Growth in broth*

*The "intermedius" phase.* After 24 hours' incubation there was a granular deposit with a fine granular supernatant. A pellicle first appeared after 3 days, becoming as well marked as with a typical *gravis* strain on the 4th or 5th day. Subcultures to tellurite media in the first 24 hours yielded only pure "*intermedius*" colonies, but scanty "*gravis*" colonies appeared in one made at 48 hours, while at 72 hours the subculture was mainly "*gravis*" in type, with few "*intermedius*" colonies. On continued incubation the number of "*intermedius*" colonies in the subcultures decreased progressively; none was detected after 14 days. In an occasional batch of broth, mutation did not occur so readily and it was observed that no pellicle appeared in its absence.

*The mutant.* After only 24 hours' incubation a slight pellicle appeared, becoming heavy and of *gravis* character after 48 hours. There was a slightly granular supernatant with a coarse flocculent deposit.

#### *Morphology*

Films from 18-hour growths of the two phases on heated blood agar, stained with methylene blue, showed differences in morphology. The "*intermedius*" phase (fig. 7) had the characteristic barred appearance, with occasional clubbing of the ends, whereas the organisms in the "*gravis*" phase (fig. 8) were shorter, the majority having only one bar and some being uniformly stained.

#### *Fermentative properties*

Tubes of Hiss's ox-serum water (pH 7.5) containing one per cent. of sucrose, starch and glucose respectively and inoculated with the organism in the "*intermedius*" and "*gravis*" phases showed in both cases failure to ferment sucrose, production of acid and clot in the starch medium within 24 hours and very slow fermentation of glucose. The reaction of the glucose medium was not reduced to pH 6.8 till after 2 days' incubation and clotting did not occur till the fifth or sixth day. Neither the "*intermedius*" nor the "*gravis*" phase developed any capacity for more rapid glucose fermentation after daily subculture or prolonged culture for a month in Hiss's glucose serum water. The *gravis* strain subsequently isolated from the case Lavin showed identical fermentative reactions and in particular the slow fermentation of glucose, a feature not shown by any other *gravis* strain isolated in the hospital.

To determine whether the fermentation of starch was due to the organism in the "*intermedius*" phase or to "*gravis*" mutants, tubes of Hiss's serum water containing one per cent. of starch were inoculated from 12-hour broth cultures shown to contain the organism in its two phases respectively. After 8 hours' incubation, subcultures from the Hiss's media were made to unheated blood-tellurite agar; thereafter 4-hourly during the first 24 hours, twice daily for the next 2 days and daily for a further 10 days, when the cultures became sterile.

The results (table) show that the changes in reaction proceeded at identical rates. After 12 hours' incubation, fermentation had occurred in the "*intermedius*" culture, with reduction of pH to 7.0,

TABLE

*A comparison of the changes in reaction of Hiss's starch-serum water inoculated with "intermedius" and "gravis" mutant phases of strain "Robins" in relation to the appearance of mutants from the "intermedius" phase.*

Time of incubation (hours)	Phase of strain "Robins" inoculated					
	"Intermedius"			"Gravis" mutant		
	pH of medium	Clotting of medium	Presence of mutants	pH of medium	Clotting of medium	Presence of mutants
0	7.6	—	None	7.6	—	Pure growth
8	7.3	—	"	7.3	—	" "
12	7.0	—	1 colony	7.0	—	" "
16	<6.8	+	Scanty	<6.8	+	" "
20	"	++	3 colonies	"	++	" "
24	"	+++	None	"	+++	" "
36	"	++++	3 colonies	"	++++	" "
48	"	++++	1 colony	"	++++	" "
60	"	++++	None	"	++++	" "
72	"	++++	"	"	++++	" "

— = no clotting

+, ++ and +++ = partial clotting

++++ = complete clotting

Presence of mutants shown by subculture to tellurite medium

when only one colony of the mutant appeared on subculture to tellurite medium in an otherwise pure "*intermedius*" growth. Partial clotting occurred after 16 hours, when subculture showed less than 0.2 per cent. of "*gravis*" mutant colonies. Complete clotting occurred after 36 hours, but the proportion of "*gravis*" mutants present became even less. It appears, therefore, that the organism in the "*intermedius*" phase could ferment starch, since if fermentation were due solely to the "*gravis*" mutant, it would proceed much more rapidly in the tube inoculated with the pure mutant. A control *gravis* strain tested in parallel fermented starch at the same rate as the "*gravis*" mutant and a control *intermedius* strain failed to ferment starch.

After 3 days' incubation, subcultures from the tube inoculated with

the "*intermedius*" phase yielded almost pure "*intermedius*" growths, with only occasional colonies of the mutant. To determine whether the non-development of the mutants was due to the fermentable carbohydrate or to the Hiss's medium *per se*, the experiment was repeated with Hiss's serum water alone and with the same medium containing added sucrose, starch or glucose. Fourteen daily subcultures to tellurite medium showed only scanty mutation in the glucose and starch media, but none in the remainder. Subcultures after the first 6 days' incubation made to nutrient broth tubes, however, developed pellicles in all cases after 4 days, with abundant "*gravis*" growths on inoculation to tellurite media. Even after 30 days there was no evidence of mutation in the Hiss's serum water, which is in agreement with the failure of serum to stimulate mutation when added to agar.

In view of the absence of mutation in Hiss's serum medium, the organism in the "*gravis*" phase was repeatedly subcultured in this medium and on inspissated serum slopes. After daily subculture for a month, there was no evidence of reversion to the "*intermedius*" phase as shown by subculture to tellurite medium and none after prolonged culture for the same period.

#### *Animal experiments*

Both the "*intermedius*" and "*gravis*" phases were virulent, as tested by subcutaneous inoculation of guinea-pigs from Loeffler slope cultures. Where recovered, the organism was always in the same phase as when inoculated, as shown by culture on unheated blood-tellurite agar, but the "*intermedius*" colonies still developed mutant papillæ on further incubation. There was therefore no evidence of mutation from "*intermedius*" to "*gravis*" phase, or vice versa, in the guinea-pig.

#### *Discussion*

In any study of the stability of types of *C. diphtheriæ* the purity of the strain must be beyond question and to obtain this by single colony selection from closely related bacterial forms may present special difficulty, as stressed by Gins and Fortner (1926). Carter emphasised the value of single-cell cultures. The difficulty then experienced in obtaining growth from single cells has now been overcome by the use of serum-enriched agar, and the improved technique for the isolation of individual cells has eliminated the possibility of contamination by adjacent organisms.

Variations occur frequently in bacterial populations, but in many cases modifications, especially those induced by environment, are transient (Dubos, 1945). Those associated with the production of papillæ appear to be more permanent and may be described as mutations. The papillary subcultures from the strain described show

all the characteristics of *gravis* strains. The mother strains differ in exhibiting *intermedius* characteristics in morphology, consistency of colonies, appearance of growth in broth and on blood-tellurite media, and the greenish colour on serum-heated-blood-agar medium (Gordon and Higginbottom). Though the difference in colonial appearance is abolished on the serum-cystine medium of Clauberg *et al.*, both phases resembling *gravis* strains, subcultures to other media still show the original colonial differences. The much smaller size of a typical *intermedius* colony on serum-cystine medium, however, suggests a relationship of the strains Robins and Lavin to the *gravis* type.

Both phases also ferment starch, a feature usually associated with *gravis* strains only. This is of significance, since most attempts to induce starch fermentation by non-starch-fermenting strains have failed and Menton *et al.* (1933) and Christison (1933) suggest that starch fermentation is a more important differential feature than cultural or morphological characteristics. Stuart (1938) described starch-fermenting strains with *intermedius* colonial appearance and morphology, but they differed from the mutating strains in that they retained these characters for 2½ years in culture and never produced a pellicle in broth. The stability of fermentative reaction is further emphasised by the unusual slow fermentation of glucose in both phases of the mutating strains; fermentation thus appears not to be influenced by changes in morphology and colonial appearance.

The tendency to mutate varies with the culture medium, being minimal on nutrient agar. It is stimulated by the incorporation of washed red blood corpuscles, but is uninfluenced by serum in spite of a great increase in growth. Mutation, therefore, is not directly related to bacterial population alone, though it proceeds much more rapidly when factors stimulating mutation and growth, as in whole blood, are both present. The use of low-temperature meat-extract broth in the agar base also stimulates mutation.

The behaviour of these mutating strains *in vivo* is of epidemiological interest. Do similar changes take place in the animal and human body, or are they only an adaptation to life on artificial media under certain conditions, as Parr and Robbins (1942) suggest occurs in the "domestication" of bacteria? No evidence of mutation was found during the short period (maximum 3 days) for which the animals survived after inoculation with the "*intermedius*" phase and Carter also found no evidence of mutation in the guinea-pig, but there is some evidence for mutation in the human body. Mair found a change to the *gravis* type in the human subject in one of the 14 cases from which mutating barred starch-fermenting strains were isolated. In one of our cases (Lavin) from which the "*intermedius*" strain was obtained initially, a *gravis* strain was subsequently isolated. This, in view of its slow glucose fermentation, appeared to be identical with the mutant obtained *in vitro* and probably arose by mutation *in vivo*, but the possibility of a *gravis* cross infection, though minimised by

cubicle nursing, cannot be completely excluded. The significance of these mutations in the epidemiology of diphtheria must await further observations on the incidence of similar strains.

### Summary

1. Two strains of *C. diphtheriæ* are described which showed colonies of *intermedius* appearance and developed papillæ, subcultures from which yielded typical *gravis* strains.

2. The strains and the mutants were cultured from single cells by an improved technique, which is described, enabling multiple single-cell isolations to be made on the surface of a solid medium by direct or dark-ground illumination.

3. The characteristics of the strains and their mutants are compared.

4. The mutation is shown to be influenced by the nature of the culture medium, being stimulated by the presence of blood pigments but not by serum.

5. No mutation was obtained in animal experiments, but evidence of mutation in the human body was adduced in one case.

We wish to thank Professor J. W. McLeod and Dr K. Zinnemann for helpful criticism, Mr J. Hainsworth for photomicrography, and Mr A. Myers and Mr W. Ivory-Hollingsworth for technical assistance.

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# TRILOCULAR HEART WITH BILATERAL ANEURYSMAL DILATATION OF THE PULMONARY ARTERIES

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(PLATE LXIX)

TRILOCULAR hearts may have either a single auricular or a single ventricular chamber and the rarity of the condition is indicated by the fact that in a series of 1000 cases of congenital heart disease collected by Abbott (1936) only 42 such cases were found. Of these, 15 had a single auricular chamber and therefore fall into the class of interatrial septal defects.

Owing to the mechanical abnormality of the circulation in these individuals, severe strain is thrown on the right side of the heart and pulmonary circulation, giving rise to an advanced form of cor pulmonale and often to marked dilatation of the pulmonary arteries. In spite of this, it is surprising to find that the average age at death is about thirty-six years, while occasional cases have survived to more than seventy years of age (Roesler, 1934; Tarnower and Woodruff, 1936; Bedford, Papp, and Parkinson, 1941; Burrett and White, 1945). That this clinical and pathological syndrome is well recognised amongst cardiologists is apparent from a recent excellent account of 53 cases, 10 of which came to post-mortem (Bedford *et al.*). It was felt, however, that on account of its general rarity the clinical and pathological features were less familiar to pathologists. The present case, being that of a hospital dispenser, was under constant observation for more than twenty years. These facts, together with the typical course and striking post-mortem findings, make the case worthy of record.

## Case report

*Clinical history.* The patient, a male dispenser, was aged 51 when he died. There was no history of cyanosis at birth, but in 1902, when aged six years, he had a "fit" and an attack of "blueness," was unconscious for three or four weeks and confined to bed for six months. He subsequently showed great improvement and during adolescence was able to walk as much as twenty to thirty miles in a day. In 1920 he had his first attack of palpitation, and although this recurred from time to time during the next nine years, the attacks were only of short duration and could be relieved by stooping. They appeared,



however, to be becoming more severe, and in 1920 one of them did not respond to simple measures. In 1932 a similar attack necessitated admission to hospital, when he was found to have marked cyanosis, dyspnoea at rest and auricular fibrillation. This and similar attacks during the next ten years were adequately controlled by quinidine therapy and the patient appears to have remained in moderately good health. By 1942 his condition had deteriorated and on admission to hospital he was found to have marked cyanosis and dyspnoea at rest, venous pulsation in the neck, slight enlargement of the liver and an erythrocytosis of 7 millions per c.mm. X-ray examination of the chest showed considerable enlargement of the left auricle and an enormous projection from the right side of the heart in the auricular region. Auricular fibrillation was confirmed by electrocardiographic examination and a bundle branch defect was observed in addition. While some general improvement followed rest and quinidine therapy, further severe attacks followed. In 1945 a severe attack of auricular fibrillation was accompanied by a hæmoptysis of about 2 oz. In 1947 he was much worse and was admitted to hospital on account of cyanosis and dyspnoea. Although he improved temporarily, his condition subsequently deteriorated, culminating in death from congestive heart failure.

*Post-mortem findings.* The general nutrition was good. Marked cyanosis of the lips was present. The pericardial cavity contained only a few c.c. of straw-coloured fluid and the sac itself appeared

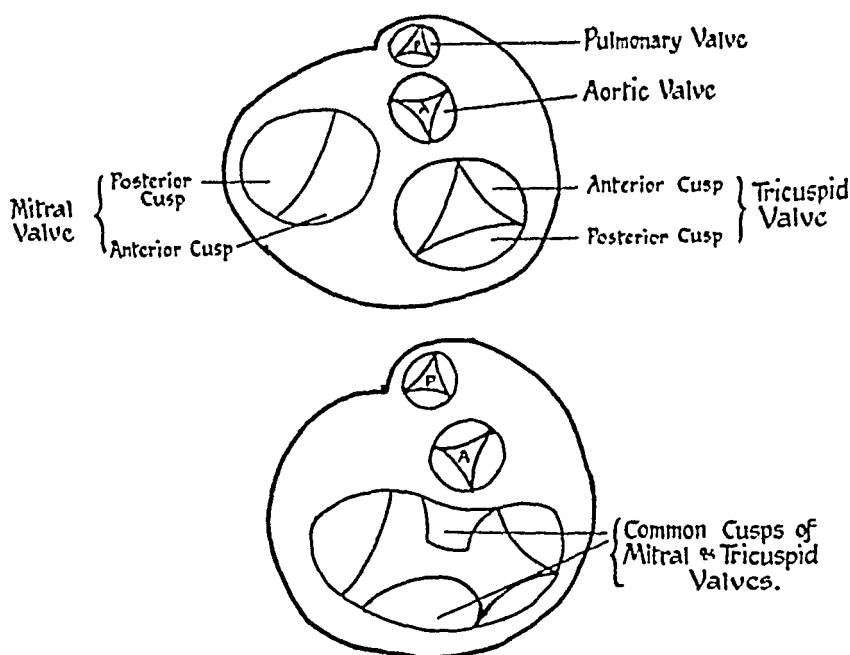


FIG. 2.—Diagram of valvular defect in present case, with normal valvular disposition (above) for comparison.

normal. The heart showed marked enlargement, especially of the right ventricle. The pulmonary conus and artery were enlarged to about twice the size of the aorta, while the single auricular cavity was dilated and its appendages were distended by ante-mortem clot.

The right auricular appendix was observed to be pointing posteriorly instead of in the normal anterior direction. No trace of an interauricular septum could be found (fig. 1), and the membranous part of the interventricular septum was also absent, though the muscular portion appeared to be normal. The division between the single auricle and the ventricles was incomplete, the medial cusp of the tricuspid valve and the anterior cusp of the mitral valve being joined to one another (fig. 2). Both these cusps were bifid, and the resulting malformed structure was firmly adherent to the upper margin of the interventricular septum (fig. 1). The remaining cusps of the tricuspid and mitral valves showed some thickening, and the right ventricle was hypertrophied and dilated. The aortic and pulmonary valves were normal. The ductus arteriosus was identified and found to be completely closed. Both pulmonary arteries, starting immediately beyond the bifurcation of the main trunk, were the seat of aneurysmal dilatation which extended for a distance of about 15 cm., the condition being more marked on the left than on the right (fig. 3). Both aneurysms were filled with a large mass of ante-mortem thrombus, parts of which appeared to have formed at different times. Marked atheroma could be seen in both pulmonary arteries, extending down to the smaller branches. The lung tissue generally was firmer in texture than normal and the bases were congested. Only one small recent infarct could be found—in the left lower lobe. The bronchial arteries were traced near the origin of the main bronchi and were found to be moderately enlarged. The peritoneal cavity contained about 300 c.c. of clear yellow fluid. The liver weighed 1850 g. and the cut surface was “nutmeg” in appearance. The spleen, which weighed 105 g., was firm and congested on section. The kidneys were deeply congested but showed no sign of present or past infarction.

*Histology. Pulmonary aneurysm.* The elastic layer is present, though markedly thinned out in some places. The adventitial layer shows increased fibrosis and patches of atheroma are present in the intima. The atheromatous plaques show both calcification and the presence of cholesterol clefts. The lumen is occupied by thrombotic material, much of which is recent and fibrinous, though some of it is old and well organised.

*Auricle.* The wall shows hypertrophic thickening, though there is also evidence of muscle-fibre atrophy with replacement fibrosis.

*Right ventricle.* The muscle fibres are thinner than normal and show other evidences of atrophy. The blood vessels are much congested and one small venule contains ante-mortem thrombus.

*Lungs.* Sections from several portions of each lung show considerable thickening of the walls of the larger pulmonary arteries, with narrowing of the lumen due to sub-intimal fibrosis. The smaller arteries are similarly affected, and one of them contains in addition an organised thrombus. Although thinning of the alveolar walls with rupture is present in places, the predominant change is fibrous

thickening, together with vascular congestion and the presence of "heart failure" cells.

*Liver.* The appearances of advanced chronic venous congestion are present.

### Discussion

While the majority of patients with patent foramen ovale suffer from no clinical symptoms during life, those with large atrial septal defects sooner or later develop a typical clinical picture.

The separation of the main atrial from the main ventricular chamber is brought about at an early stage of development by the growth and fusion of the dorsal and ventral endocardial cushions. Later the atrium becomes divided by the interatrial septum which results from the fusion of the septum primum and septum secundum. In the normal interatrial septum, the septum secundum is represented by the portion above the foramen ovale, the septum primum by the portion below it. It follows that some idea of the nature of the patency can be obtained by observing its position in the septum relative to the position of the foramen ovale. In the present case careful search failed to reveal the presence of any portion of the septum. A prominent band of tissue on the anterior atrial wall was examined histologically and found to consist of cardiac muscle, and accordingly regarded as a limbic band. The portion of the atrium between the entry of the superior vena cava and right pulmonary veins was given particular attention, though it was realised that in congenital disease of the heart there is frequently an abnormal entry of the pulmonary veins into the right atrium. It has been stated that failure of fusion of the endocardial cushions always results in an abnormality of the tricuspid or mitral valves (Costa, 1931; Rogers and Edwards, 1948). In the present case both the medial cusp of the tricuspid and the anterior cusp of the mitral were bifid. This valvular malformation therefore indicated that there was failure of fusion of the endocardial cushions in addition to maldevelopment of the interatrial septum (fig. 2).

Atrial septal defect is the most frequent congenital cardiac malformation and occurs either alone or in association with a variety of other cardiac abnormalities. It has two very interesting characteristics. First, in contradistinction to other congenital heart lesions, it is only very rarely followed by the development of subacute bacterial endocarditis. Second, it is the only congenital heart lesion which is at all frequently associated with mitral stenosis and therefore also with auricular fibrillation (Wolbach and Abbott, 1915; Lutembacher, 1916). The present case was unusual in that there had been auricular fibrillation over a long period, yet without any evidence of mitral stenosis *post mortem*.

Clinically, cyanosis may never become manifest in congenital heart disease. On the other hand it may dominate the clinical picture throughout, or appear only in the later stages ("cyanose tardive").

## TRILOCULAR HEART AND PULMONARY ANEURYSM

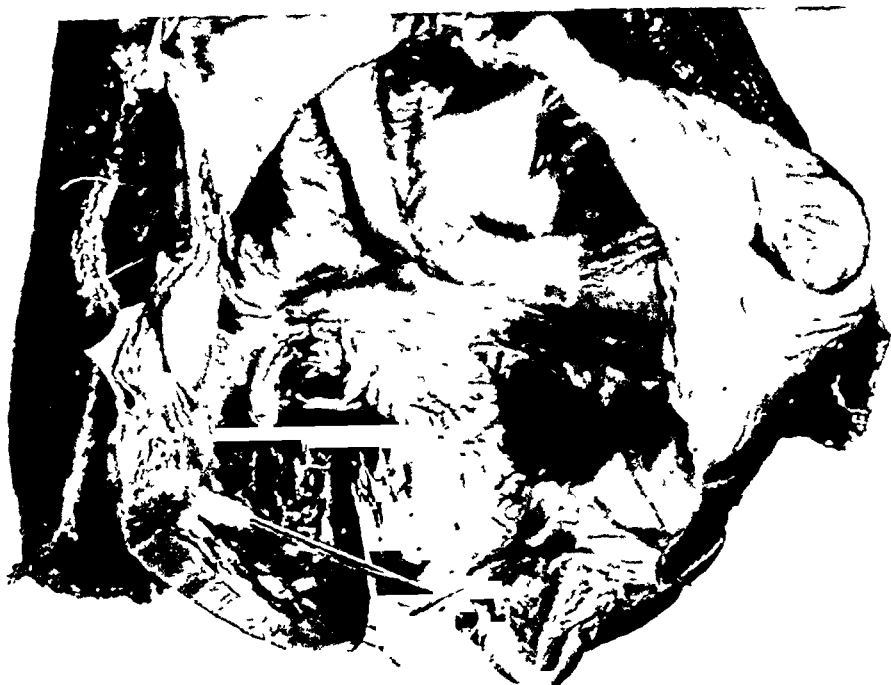


FIG. 1.—View of heart from above; it has been opened posteriorly. Note broad band of tissue in anterior auricular wall, also common auricular cavity. The bifid cusps of the tricuspid and mitral valves are seen adherent to the underlying inter-ventricular septum. The left ventricle is held open by a glass rod. Note that the right auricular appendix is pointing posteriorly instead of anteriorly.



FIG. 3 —Bifurcation of pulmonary artery showing large laminated ante-mortem thrombi in the dilated pulmonary arteries, especially the left.



Cases of interatrial septal defect fall into the "cyanose tardive" group and the typical course is well illustrated by the present example. The harmful effects of the abnormality are due to the passage of a proportion of the circulating blood from the left to the right auricle. Proof that this occurs has been recently forthcoming in the work of Stead and Warren (1947) and Howarth, McMichael and Sharpey-Schafer (1947), who demonstrated by means of intra-cardiac catheterisation that, at rest, oxygenated blood flows through the septal defect from left to right. As a result of this shunt a great strain is thrown on the right side of the heart and the right ventricle undergoes hypertrophy in an attempt to deal with the larger volume of blood reaching it. In some cases this enlargement has progressed until, in the latest stages of the disease, the right ventricle is dealing with an amount of blood three times as great as that dealt with by the left. In the earlier stages there is no cyanosis, since the shunt takes place from left to right, and a proportion of the blood is thus being passed twice through the lungs for oxygenation. While this mechanism continues the patient remains moderately well in spite of the cardiac enlargement. Indeed, in the present case we have evidence that the exercise tolerance at twenty years of age was very satisfactory, for he was in the habit of walking as much as twenty or more miles a day. Cardiac decompensation first revealed itself in the form of exertional dyspnoea and transient cyanosis, though the exact date when this occurred was uncertain. It would appear that the first really serious attack of cyanosis was associated with auricular fibrillation fifteen years before death. Auricular dilatation was present at this time, and the cyanosis in these cases of "cyanose tardive" is merely a signal that the shunt has become reversed and that the blood is now passing from right to left in the auricular chamber. In the present case rest and quinidine restored the normal auricular rhythm, with consequent improvement in the cyanosis. In later years further attacks of auricular fibrillation became progressively more difficult to control, until a state was reached in which cyanosis was more or less permanent and the auricular fibrillation was resistant to quinidine therapy. By this time cardiac failure was well established, and it is a remarkable feature that in cases of interatrial septal defect failure rarely appears until the third decade of life, in spite of the gross circulatory abnormality and the severe strain imposed on the right side of the heart. Even more remarkable, perhaps, is the fact that, as in the present instance, patients may survive up to fifteen years after the failure first manifests itself.

This case also illustrates excellently some of the other adverse factors which may come into play. As a result of increased pulmonary pressure atheromatous degeneration was produced in the already dilated pulmonary arteries and the long-continued anoxia had given rise to a severe degree of erythrocytosis. Thus to stagnation in a dilated pulmonary artery there was added, as a result of cardiac

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## EBSTEIN'S ANOMALY OF THE TRICUSPID VALVE

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(PLATES LXX AND LXXI)

CONGENITAL malformation of the tricuspid valve is rare. In a series of 1000 cases of congenital heart disease, Abbott mentions 13 instances of stenosis and 25 of tricuspid atresia, many of them associated with other developmental defects. Nevertheless a great variety of tricuspid anomalies have been described (Herxheimer, 1910; Abbott, 1946). Ebstein (1866) was the first to report a congenital displacement of the tricuspid valve associated with patency of the foramen ovale. Since then, 15 similar cases have appeared in the literature and these have been reviewed by Yater and Shapiro (1937-38). Two further cases have recently been reported by Zink (1937) and Bauer (1945).

The essential feature of Ebstein's anomaly is the downward displacement of a malformed tricuspid valve into the right ventricle. Whereas the anterior leaflet is in part attached to the annulus fibrosus, the remainder arises with the middle and posterior leaflets from the right ventricular wall and interventricular septum. The right side of the heart is thus so divided that part of the right ventricle functions with the right auricle. The individual leaflets are thickened and sometimes undifferentiated. Incompetence results from short chordæ tendinæ and poorly developed papillary muscles. Both right auricle and right ventricle are dilated and hypertrophied. The foramen ovale is usually patent (16 out of 19 cases), possibly as a result of the increased pressure within the right auricle. The Eustachian and Thebesian valves are often well developed and may help to prevent the regurgitation of blood into the venæ cavæ and the coronary sinus. The main features of the anomaly are represented diagrammatically in fig. 1.

There is no characteristic clinical picture, as this depends on the degree of tricuspid incompetence and the volume of blood shunted through the foramen ovale. Dyspnœa, cyanosis and clubbing of the fingers may be marked or absent. The heart is enlarged and X-ray examination shows that the right chambers are mainly affected. A long systolic murmur maximal in the third and fourth left intercostal



On admission the most noticeable feature was the pronounced cyanosis with surprisingly little dyspnoea. There was no clubbing of the fingers. The pulse was regular at 80 beats a minute, with normal arteries and fundi. The blood pressure was 140/90 mm. Hg., but had been 170/110 when measured in the out-patient department a week before. Venous congestion in the neck was over three inches; the veins could be emptied and did not pulsate excessively. There was moderate oedema of the ankles and rales were present at both lung bases, but there was no ascites or pleural effusion. The liver was tender and palpable two inches below the right costal margin; it did not show expansile pulsation. A forcible apex beat was palpable  $4\frac{1}{2}$  inches from the mid-line in the 5th intercostal space. The percussion note was normal at the base and the right border of cardiac dullness was 2 inches to the right of the midline. Apart from an accentuated second sound over the pulmonary area the heart sounds were normal and there were no murmurs. The following investigations were made at this time.

*X-ray.* Enlargement of both ventricles and auricles without prominence of conus arteriosus. Lung fields congested (fig. 2).

*E.C.G.* Low voltage curves, normal rhythm and right ventricular preponderance (fig. 3).

*Blood.* Hb 106 per cent., R.B.C. 6,500,000, W.R.—: Serum anti-complementary.

*Circulation time.* Decholin arm-tongue time 23 seconds. Ether arm-lung time 12 seconds, but end-point indefinite. The cyanosis and high venous pressure disproportionate to the dyspnoea being still unexplained, it was considered that an estimation of the vital capacity might help to exclude a pulmonary cause, and that direct measurement of the venous pressure would establish this factor beyond doubt.

*Vital capacity:* 2370 c.c. (normal 3500 c.c.).

*Direct measurement of venous pressure* (Kendrew, 1926): 14 cm. of saline, with normal respiratory excursion and no excessive systolic pulsation.

The congestive heart failure was at first considered to have been precipitated by hypertension and coronary disease, but as this did not account for the very high venous pressure and marked cyanosis, other factors were discussed. The slight dyspnoea with high venous pressure was consistent with a diagnosis of constrictive pericarditis, but the other clinical and radiological features of this condition were absent. Chronic cor pulmonale could produce the congestive failure, cyanosis, polycythaemia and loud pulmonary second heart sound, and the X-ray and electrocardiographic evidence of right ventricular hypertrophy, but the absence of signs and symptoms of pulmonary disease, the poor response to oxygen therapy and the relatively normal vital capacity did not support this diagnosis. Congenital heart disease was suggested by the right ventricular hypertrophy and cyanosis, but the absence of murmurs and of any characteristic cardiac configuration made a certain diagnosis impossible.

Complete rest, restricted fluids with low salt intake, digitalis and oxygen therapy produced no clinical improvement. A few hours after a small venesection (300 c.c.) the classical symptoms and signs of a large pulmonary embolism occurred, and later those of infarction of the left lower lobe. The source of this embolus was probably the calf veins, as thrombosed and tender veins were subsequently felt in both legs. Treatment with heparin and Dicoumarol was begun at once and controlled by frequent prothrombin estimations, but after ten days this therapy was discontinued in order that a large left pleural effusion could be tapped. Frank haematuria occurred at this time, although the prothrombin and clotting times were normal. The patient continued desperately ill, with repeated haemoptyses and recurring left pleural effusion, and she died three weeks after the pulmonary embolism.

## EBSTEIN'S TRICUSPID VALVE ANOMALY

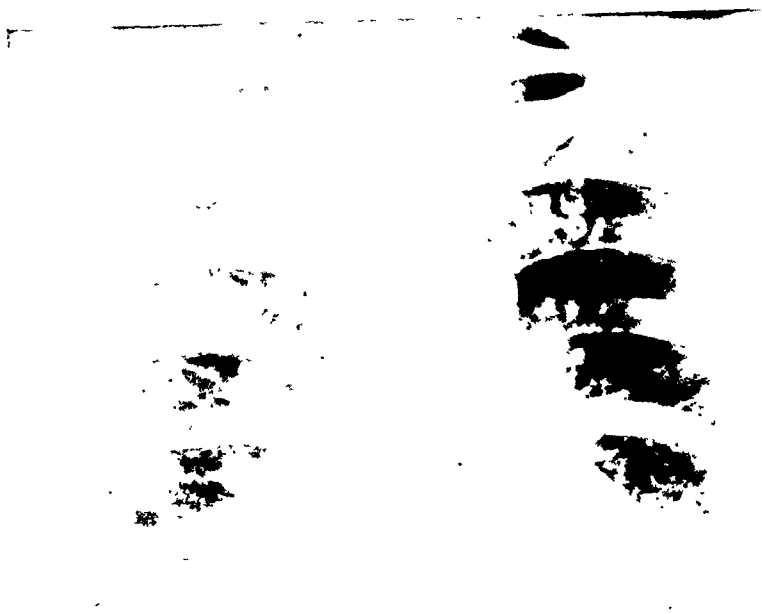


FIG. 2.—X-ray showing enlargement of both ventricles and auricles (without prominence of the conus arteriosus) and congestion of the lung fields.

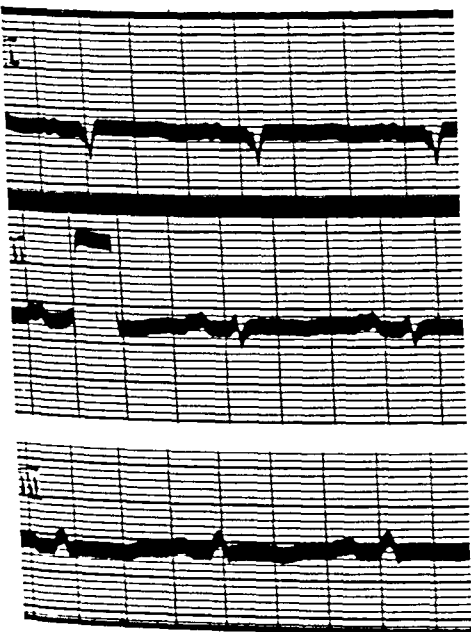


FIG. 3.—E.C.G. showing low voltage curve, normal rhythm and right ventricular preponderance.

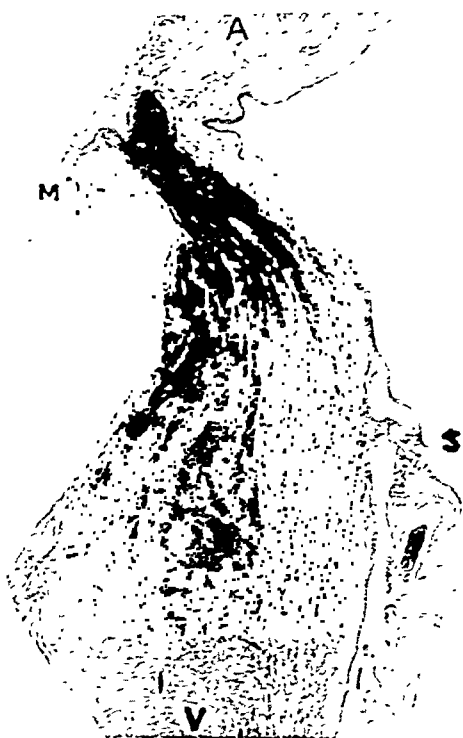


FIG. 5.—Section through interventricular septum. A = interauricular septum; M = mitral valve; S = displaced septal cusp of tricuspid valve; V = interventricular septum. Reticulin stain.  $\times 5$ .



*Autopsy report*

The autopsy was performed by Professor G. R. Cameron 25 hours after death. Externally, the features of note were the cyanosis of the lips and mucous membranes and the swelling and œdema of both legs, especially the right.

*Heart.* The heart was enlarged (425 g.). Hypertrophy and dilatation of the right side of the heart were particularly obvious. The pericardial sac contained 5 oz. of clear, straw-coloured fluid but there was no evidence of pericarditis. There was a moderate amount of subepicardial fat and the coronary veins were congested.

*Valves.* The tricuspid valve was found to be displaced, malformed and obviously incompetent. The true atrio-ventricular opening was widely dilated, being 15 cm. in circumference. The actual orifice of the valve was also dilated and the line of attachment of the cusps was displaced downwards into the right ventricle. The opening was bounded medially by a small incompetent septal cusp and anteriorly and laterally by a single membrane which represented the fused anterior and posterior cusps. The septal cusp arose from the inter-ventricular septum and ventricular wall 3 cm. below the annulus fibrosus, its normal line of attachment. This cusp was smaller than normal, irregularly roughened and thickened and the chordæ arising from it were mostly short, thin and inserted directly into the ventricular wall without the intervention of papillary muscles (fig. 4). The cusp was, in consequence, bound down to the inter-ventricular wall and clearly incompetent. The anterior and posterior cusps were fused at their adjoining edges, forming a single membrane arising in part from the inter-ventricular wall and in part from the wall of the ventricle below the atrio-ventricular groove.

Apart from a slight Mönckeberg's ring in the aortic valve, the other valves showed no abnormality and were competent.

*Chambers.* The right auricle was dilated and its wall was thin. The dilatation extended to the auricular appendage, which contained in addition a number of small ante-mortem clots adherent to the muscle. The inter-auricular septum showed a slit-like patency of the foramen ovale. The opening present was 1.5 cm. wide and passed obliquely forward through the septum into the left auricle. The opening of the superior vena cava was guarded by a well-marked Eustachian valve. The coronary sinus was dilated but no definite Thebesian valve was present. Due to the displaced position of the tricuspid valve a portion of the right ventricle was "atrialised", i.e. was functionally continuous with the right auricle.

The left auricle was free from clot. The opening in the anterior border of the foramen ovale was guarded by a crescentic valve-like flap.

The left ventricle was normal in size and shape. The muscle of the left ventricle showed patchy fibrosis at the apex and on its septal surface.

The walls of all four chambers felt firmer and more fibrous than normal.

*Vessels.* The aorta and pulmonary artery were in normal relationship with one another and with their respective ventricles. The aorta was narrowed throughout its length and showed moderate atheroma of its abdominal portion. A small atheromatous plaque considerably narrowed the anterior descending branch of the left coronary artery but complete occlusion had not occurred.

A paradoxical embolus was found in the left common iliac artery. This was a loose ante-mortem clot about 0.5 cm. in diameter and 5 cm. in length lying free within the left common iliac artery and extending into the external iliac artery. The source of this embolus appeared to be a large ante-mortem thrombus firmly attached to the wall of the left femoral vein and extending down the leg to the commencement of the popliteal vein.

Evidence of embolism and infarction was also found in the lungs, spleen and both kidneys. An infarct in the lower lobe of the left lung occupied the whole lobe and was accompanied by a massive fibrinous pleurisy in the left pleural cavity. The remaining organs were congested but showed no other pathological abnormality.

Microscopic examination showed no evidence of rheumatic disease in the myocardium or in the malformed valve. The myocardium of both ventricles and of the right auricle showed widespread fibrosis of the type associated with long-standing ischæmia. The lumen of the left descending coronary artery was partially filled by an atheromatous plaque containing microscopic foci of calcification. The smaller vessels throughout the myocardium showed hyaline thickening of their walls and great reduction of their lumina.

The cause of death was myocardial failure associated with chronic myocardial fibrosis and tricuspid insufficiency, the latter being caused by a congenital abnormality of the tricuspid valve (Ebstein's anomaly). Pulmonary embolism and infarction, infarction of the spleen and kidneys and paradoxical embolism involving the left common and external iliac arteries originating from the left femoral and popliteal veins were also found.

### *Summary*

Previously reported cases of Ebstein's anomaly of the tricuspid valve are reviewed in summarised form and a further case showing in addition the phenomenon of paradoxical embolism, is reported.

We wish to record our grateful thanks to Professor G. R. Cameron, Professor H. P. Himsworth and Dr M. L. Rosenheim for encouragement and permission to report this case and for their help in preparing the manuscript.

EBSTEIN'S TRICUSPID VALVE ANOMALY



FIG. 4.—Showing displaced and atresic septal cusp and patent foramen ovale.



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576 . 8 . 077 . 34 : 576 . 851 . 48 (*Bact. coli* var. *neapolitanum*)

## SLIDE AGGLUTINATION OF *BACTERIUM COLI* VAR. *NEAPOLITANUM* IN SUMMER DIARRHŒA

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INVESTIGATIONS made during 1943 (Bray, 1945) showed that a particular serological type of *Bact. coli* was recoverable from 95 per cent. of cases of summer diarrhœa or non-specific gastro-enteritis of infants. Originally these strains were detected by taking advantage of the delay which, under certain conditions, they showed in the fermentation of maltose. In the present investigation, carried out during 1945, the method of slide agglutination was used for the detection of these strains, as the earlier method of isolation proved unreliable. The results suggest that this method might be of practical value for the laboratory confirmation of the clinical diagnosis of a particular type of gastro-enteritis.

### *Clinical material*

During 1945, 40 cases were diagnosed as gastro-enteritis in infants of a mean age of 5 months, the cases being spread over the period February to October. Fæcal samples from these and from 80 non-diarrhœal controls from the same wards were examined, the cases selected being from bottle-fed infants under one year of age.

In three-quarters (28) of the diarrhœal cases the disease was of a severe type, i.e. requiring parenteral fluids, and there were 11 deaths (28 per cent.). The characteristic smell noted by Beavan (1944) was obviously present, although not especially sought for, in 17 cases. In nearly half the cases (18) it was considered that the disease had been acquired during the infant's stay in hospital.

### *Post-mortem findings*

Six autopsies were performed and the findings were characteristically meagre. In 2 cases there was marked monilial œsophagitis. There was macroscopic evidence of fatty changes in the liver in 4. In 3 cases partially digested blood was present in the stomach, but in none were there significant changes in the mucosa of the intestines. Œdema of the brain was noted in 4 cases. Agglutinable strains of *Bact. coli* var. *neapolitanum* were isolated from all these cases.

## Methods

Faecal samples from each case were plated on desoxycholate-citrate agar and MacConkey's agar. The former was examined for non-lactose fermenters with the usual lack of significant findings, except in one case where *Bact. sonnei* was isolated. From the MacConkey's agar a single pink colony was picked and tested on a slide against the antiserum which had been used in the 1943 investigations (Bray, 1945) and which had been preserved in the refrigerator. In the selection of a colony, one which was transparent to transmitted light and not obviously rough or mucoid was taken. Otherwise, apart from choosing one that was well isolated, the colony was taken at random. Before use the serum was diluted with saline 1 in 10 and the final dilution on the slide was approximately 1 in 400. In spite of this there were few doubtful reactions. Agglutination was, in the positive cases, usually immediate and unequivocal and of the granular kind, like that given by an O strain.

The identity of all agglutinating strains was later confirmed by tube agglutination and the testing of fermentative reactions against sucrose, salicin and maltose, and by motility tests. After picking the colony the MacConkey plate was laid aside on the bench and examined from time to time for the growth of *Monilia*. It had been previously noted that an easily recognisable growth of *Monilia* appeared in about 4 days in the form of discrete granular white domes on the site of the thickest inoculum, where the coliform growth was confluent.

## RESULTS

Table I shows the number of isolations of *Bact. coli* var. *neapolitanum* resulting from the investigations of 1943 and 1945.

TABLE I

*Isolations of Bact. coli* var. *neapolitanum* (B.C.N.) from cases of gastro-enteritis and from controls

Year	Gastro-enteritis			Controls	
	No. of cases	Isolation of B.C.N. (per cent.)	Mortality (per cent.)	No. of controls	Isolation of B.C.N. (per cent.)
1943	50	95.0	30	100	4
1945	40	87.5	28	80	4

In the gastro-enteritis samples the slide agglutination was positive in 35 cases. Later tests showed these strains of *Bact. coli* to be of the same type as those reported in the earlier communication (Bray). In the controls, agglutination was positive 5 times, 3 of the strains being of the same type as those from the diarrhoeal cases, the other 2 of a different type as judged by fermentation and motility tests; in other words there were 2 false-positive agglutinations. Considering its dilution of 1 in 400, therefore, the serum seemed to show considerable discrimination.

With regard to the *Monilia*, these yeasts grew in 15 of the diarrhoeal cases (37 per cent.) and in 18 (22.5 per cent.) of the controls.

Although *Monilia* is found rather frequently in the mouths of normal adults (14 per cent., Todd, 1937) it seems that there is a close connection between the presence of *Monilia* in the oral cavity (Anderson, Sage and Spaulding, 1944) and perhaps in the faeces (Ludlam and Henderson, 1942) and the existence of clinical thrush in new-born infants. It is likely that our figures represent a high incidence of thrush infection in the wards, although we have no clinical data to confirm this. The point to note is that there is little difference in the incidence of *Monilia* in the faeces of the diarrhoeal and non-diarrhoeal cases.

### *Identity of strains*

It has been noted that in isolating the strains the slide agglutinations were done with the antiserum prepared in 1943. During 1946 a further antiserum was prepared in the rabbit against one of the strains (G.R.) isolated in 1945. Thirteen of the 1945 strains, which had been preserved in nutrient broth at room temperature, were tested against it by the tube-agglutination method. The results were that 10 of the strains were agglutinated to titre, 2 had become rough, and 1 strain which in the meantime had lost its agglutinability to the 1943 serum also failed to react with the 1946 serum. A strain (Lindsay) which had been isolated in 1943 and preserved was also agglutinated, to titre, by the 1946 serum. While the type of agglutination on the slide was granular, in the tubes it was semi-flocculent and showed some adherence to the sides in the lower part of the tube.

Identity of the 1943 and 1945 strains was further supported by a small number of absorption tests which were carried out in the manner described by Mackie and McCartney (1948). The 1943 and 1946 sera were each absorbed with a strain of the homologous and heterologous organism and then re-tested for agglutination against the same 1943 and 1945 strains. It was found in each case that the agglutinins had been removed from the sera. These results are shown in tables II-IV.

The various serological tests may thus be summarised :

- (1) Serum 1943 tested against Lindsay (1943) in order to determine titre. Approximately 1 in 8000.
- (2) 38 strains (1945) isolated by slide agglutination with the aid of 1943 serum (results confirmed by tube agglutination).
- (3) Serum 1946 prepared against G.R. (1945). Titre 1 in 16,000. This serum was prepared in a rabbit of the same strain and the same dosage of bacterial suspension was used as in the preparation of the 1943 serum.
- (4) 13 strains of 1945 origin tested by the tube-agglutination method against serum 1946. 10 strains were agglutinated to titre.
- (5) Lindsay (1943) shown to agglutinate to titre with 1946 serum.
- (6) Serum 1943 absorbed with Lindsay (1943) and G.R. (1945) and re-tested against these strains. The agglutinins for both strains

were shown to have been completely absorbed by each. Similar absorption of the 1946 serum gave identical results.

TABLE II

*Absorption of 1943 serum by strain G.R. (1945) and strain Lindsay (1943)*

Final dilution	1 in 1000	1 in 2000	1 in 4000	1 in 8000	1 in 16,000	1 in 32,000	Control
Unabsorbed serum							
G.R. suspension . . .	...	+	+	+	+	-	
Lindsay suspension . .	...	+	+	+	±	-	-
Serum absorbed with Lindsay							
G.R. suspension . . .	-	-	-	-	-	-	
Lindsay suspension . .	-	-	-	-	-	-	-
Serum absorbed with G.R.							
G.R. suspension . . .	...	-	-	-	-	-	
Lindsay suspension . .	...	-	-	-	-	-	-

TABLE III

*Absorption of 1946 serum with the homologous strain G.R. and strain Lindsay (1943)*

Final dilution	1 in 2000	1 in 4000	1 in 8000	1 in 16,000	1 in 32,000	Control
Unabsorbed serum						
G.R. suspension . . .	+	+	+	+	-	
Lindsay suspension . .	+	+	+	-	-	-
Serum absorbed with G.R.						
G.R. suspension . . .	-	-	-	-	-	
Lindsay suspension . .	-	-	-	-	-	-
Serum absorbed with Lindsay						
G.R. suspension . . .	-	-	-	-	-	
Lindsay suspension . .	-	-	-	-	-	-

(7) Two other 1945 strains (Fisher and Alyott) were used to absorb the 1946 serum. On re-testing the serum against these two strains agglutinins were found to have been removed as in (6). Unfortunately

we have no record that this absorbed serum also failed to agglutinate strain G.R., so that the identity of these two strains with G.R., though probable, is not proved.

TABLE IV

*Absorption of 1946 serum with strains Fisher (1945) and Alyott (1945)*

Final dilution	1 in 500	1 in 1000	1 in 2000	1 in 4000	1 in 8000	1 in 16,000	Control
Serum unabsorbed							
Fisher unboiled suspension .	+	+	+	+	±	Tr	—
Alyott unboiled suspension .	+	+	+	+	+	—	—
Fisher boiled suspension .	...	+	+	+	+	Tr	—
Alyott boiled suspension .	+	+	+	+	+	Tr	—
Serum absorbed with Fisher							
Fisher suspension . . .	+	+	Tr	—	—	—	—
Alyott suspension . . .	+	±	—	—	—	—	—
Serum absorbed with Alyott							
Fisher suspension . . .	Tr	—	—	—	—	—	—
Alyott suspension . . .	—	—	—	—	—	—	—

In these experiments broth cultures grown at 22° C. overnight and diluted with normal saline were used. The water-bath was kept at 56° C. and the results read after overnight incubation.

#### *Nature of antigens*

Little work has been done to elucidate the nature of the agglutinogens present in these bacteria. Broth cultures of G.R., Fisher and Alyott were boiled for ten minutes in the water-bath and tested against the 1946 serum. No difference was found in titre whether using the boiled or unboiled cultures. This indicated that H agglutinogens were not responsible for the agglutination. On the other hand on heating the serum to 70° C. for 10 minutes, at which temperature O agglutinins are labile, the titre was not shown to be altered when tested against a single strain.

#### *Alpha agglutinins of Stamp and Stone (1943-44)*

These agglutinins are present in the sera of some rabbits, possibly as the result of infection with paracolon bacilli, and their presence has been found to lead to false positive reactions in diagnostic slide-agglutination tests. It was considered possible that these agglutinins might also be present in our sera. An alpha-agglutinating serum

and also an appropriate bacterial strain were procured and a number of slide agglutinations carried out. No evidence was found of the presence of agglutinins of this type in the antisera we had prepared or of the corresponding agglutinogens in the *Bact. coli* var. *neapolitanum* strains.

### *Action on carbohydrates*

A characteristic of the strains isolated in 1943 was that of late maltose fermentation under the conditions of the test as defined in the earlier communication. This, in contrast, was not a property of the strains isolated in 1945. Only one of the 13 strains tested against maltose showed any delay in fermenting this carbohydrate. It is clear, therefore, that this is not a characteristic which can be relied on in the primary isolation of these strains.

With regard to sucrose and salicin the findings were similar to those of 1943. With both these substances fermentation was demonstrated if the tubes were kept in the incubator for 4 days. All the strains, like those of 1943, were non-motile.

### DISCUSSION

Considering the multiplicity of antigenic types of *Bact. coli* normally found in the gut of the same and different persons (Boivin, Corre and Lehout, 1942; Wallick and Stuart, 1943), and considering also that positive slide agglutinations were got so easily from the diarrhoeal cases by the picking of a single colony, it is strongly suggested that, in certain cases of gastro-enteritis, the coliform flora of the gut becomes replaced by a single type of these bacteria—a type which is found but rarely in normal children.

As different types of *Bact. coli* appear very alike on a MacConkey plate, the alteration from the normal is not apparent until the bacteria are investigated serologically.

Thus one is tempted to postulate that there exists a type of gastro-enteritis in bottle-fed infants which has a high mortality rate and an association with warmer weather, and is marked clinically in many cases by a seminal smell and bacteriologically by a change in the *Bact. coli* flora of the gut.

This type of gastro-enteritis might be separated on the one hand from infections due to *Bact. sonnei*, which appear to have a greater incidence in older children and are marked clinically by the presence of blood and pus in the faeces, and on the other from the cases of gastro-enteritis, including neonatal diarrhoea, which are distinguished by no special features, clinical or bacteriological.

What aetiological connection, if indeed any, *Bact. coli* var. *neapolitanum* has to this suggested group of cases remains, in the absence of the demonstration, for example, of an enterotoxin, entirely *sub judice*.

## SUMMARY

The technique of slide agglutination has been applied to the detection of a serologically homogeneous type of *Bact. coli* var. *neapolitanum* in cases of summer diarrhoea.

Results were positive in 87.5 per cent. of the cases, and, if 2 false positive results are excluded, in only 4 per cent. of the controls.

The frequency of Monilia in the faeces of these cases was also investigated by culture.

To Mr John Stevenson, for his valuable technical assistance, our thanks.

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## POSTSCRIPT

Since this communication went to press, our attention has been drawn to a paper by Giles and Sangster (1948) in which it is stated that two types of *Bact. coli* were recovered in an epidemic of infantile gastro-enteritis occurring in 1947 at Aberdeen. One of these types, which was identical biochemically and serologically with strains Lindsay (1943) and G.R. (1945), was recovered in 94.7 per cent. of cases. Both the Aberdeen strains were sensitive to streptomycin *in vitro* (Giles, 1948).

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# STARCH-FERMENTING GELATIN-LIQUEFYING CORYNEBACTERIA ISOLATED FROM THE HUMAN NOSE AND THROAT

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IN routine examination of nose and throat swabs for *Corynebacterium diphtheriæ*, organisms morphologically similar to *C. diphtheriæ* var. *gravis* are occasionally isolated which ferment glucose, maltose and starch rapidly, but are sufficiently atypical to call for further investigation. They liquefy a gelatin slope slowly at room temperature and on intradermal injection into guinea-pigs produce ulcerative lesions not neutralised by diphtheria antitoxin. On subcutaneous injection of the organisms, however, diphtheria antitoxin will sometimes protect a control guinea-pig from death.

## LITERATURE

There are many references in the literature to diphtheria-like organisms associated with morbid conditions of the throat and neighbouring parts which were pathogenic for guinea-pigs and were neutralised incompletely, if at all, by diphtheria antitoxin (Ruediger, 1903-06; Hamilton, 1904, 1907; Hamilton and Horton, 1906; Havens, 1920; Wells, 1932). Biochemical details, however, are not given in the description of most of these organisms and it is not possible to be certain that they were of the type under consideration.

Gilbert and Stewart (1926-27) investigated a series of gelatin-liquefying corynebacteria which were isolated from thirty-one patients over a period of six years. They named their organism *Corynebacterium ulcerans*; it fermented glucose, maltose and dextrin in serum water and failed to reduce nitrates to nitrites. Starch fermentation was not tested.

Mair (1928) described the reactions in guinea-pigs injected intradermally and subcutaneously with a strain which Barratt (1933) later found to ferment starch and liquefy gelatin. With this strain intradermal injection led to necrosis and pus formation at the site of injection in both test and control guinea-pigs, even if the control animal was given as much as 2000 units of antitoxin. If the organisms were given subcutaneously, an unprotected guinea-pig died in three days with the usual signs of diphtherial infection, but a guinea-pig which had received 1000 units of antitoxin on the preceding day survived, though an abscess developed at the site of inoculation.

Barratt (1933) investigated the strain isolated by Mair and four strains which she herself isolated, and compared them with diphtheroids of her groups III and V (Barratt, 1924-25) and with a strain of *Corynebacterium ovis*. She showed that subcutaneous injection of Mair's strain 2255 into a guinea-pig produced marked congestion of the suprarenals, but that with the other starch-fermenting gelatin-liquefying strains the suprarenals were practically unaffected and the post-mortem appearances were similar to those in guinea-pigs dead after the inoculation of a culture of *C. ovis* (see also Carne, 1939).

Petrie and McClean (1934), who studied the toxins produced by *C. diphtheriæ* and *C. ovis* and concluded that there was no relationship between them, also investigated the toxins produced by some of the strains used by Barratt (1933) in her investigation. Their observations showed that the Mair strain produced a toxin immunologically identical with diphtheria toxin and a toxic element not neutralised by diphtheria antitoxin. The work also indicated that the toxic constituent of the Mair strain not neutralisable by diphtheria antitoxin had an affinity with the toxin of *C. ovis*.

### EXPERIMENTAL OBSERVATIONS

Six starch-fermenting gelatin-liquefying strains of *Corynebacterium* were isolated at Oxford during twelve months. These were investigated together with Mair's strain, the Stead and Revell strains studied by Barratt (1933) and by Petrie and McClean, and 17 strains from other sources. Of these 26 strains, 24 were from nose or throat swabs, one from an "empty" milk bottle, and one from the milk of a cow with acute mastitis. In this paper the morphology, cultural characteristics and biochemical reactions of these strains are briefly described and compared with the reactions of fifteen strains of *C. diphtheriæ* (5 *gravis*, 5 *intermedius* and 5 *mitis*), four strains of *C. ovis*, five other named types of *Corynebacterium* from the National Collection of Type Cultures, and five corynebacteria of diverse biochemical activities that were isolated here, four from routine nose and throat swabs and one from the skin. Animal experiments were performed with all the starch-fermenting gelatin-liquefying strains, with three strains of *C. diphtheriæ* and five of the other corynebacteria.

#### *Morphology*

Stained with methylene blue after 24 hours on blood agar at 37° C., these organisms appeared as irregularly arranged, irregularly staining pleomorphic rods with rounded ends and slightly convex sides. Club, coccoid and long filamentous forms were more common after 48 hours' incubation. There were few metachromatic granules. The organisms were Gram+, non-motile and non-acid fast; they did not form spores and were non-capsulated.

#### *Cultural characteristics*

The cultural characteristics of the starch-fermenting gelatin-liquefying strains are summarised in table I and compared with those of *C. diphtheriæ* var. *gravis* and *C. ovis*.

#### *Biochemical reactions*

The following investigations were carried out on all the strains examined in the manner described in the appendix.

(1) Fermentation reactions in glucose, maltose, sucrose, starch, dextrin, glycerol, mannitol, galactose and trehalose. (2) Indole

TABLE I  
*Cultural characteristics which help to distinguish the starch-fermenting, gelatin-liquefying strains from C. ovis and C. diphtheriae var. gravis*

	<i>C. diphtheriae</i> var. <i>gravis</i>	Starch-fermenting gelatin-liquefying <i>corynebacteria</i>	<i>C. ovis</i>
Growth on a Loeffler serum slope aerobically at 37° C.	Growth not so abundant as that of starch-fermenting, gelatin-liquefying strains and paler in colour	Heavy, creamy growth after 24 hours' incubation	Yellowish-white friable growth after 24 hours' incubation
Growth on blood agar plate aerobically at 37° C.	Colonies 1-2 mm. in diameter after 24 hours' incubation. Low convex, whitish, opaque, with a finely matt surface and slightly irregular edge. Colonies usually surrounded by a small zone of hemolysis. Colonies 2-5 mm. in diameter after 48 hours' incubation. Whitish, opaque centre and more translucent periphery, flattened, low convex, irregular edge and finely matt surface	Colonies 0.5-1 mm. in diameter after 24 hours' incubation. Low convex, yellowish-white, opaque, with a matt surface. Slightly irregular edge. Most strains surrounded by a small zone of hemolysis. Colonies 1-6 mm. in diameter after 48 hours' incubation; central area slightly raised	Yellowish-white, opaque, convex colonies, about 1 mm. in diameter after 24 hours' incubation. Matt surface. Colonies increased only slightly in size after a further 24 hours' incubation. Colonies of three of the four strains examined were surrounded by small zones of hemolysis
Growth on whole unheated blood tellurite agar plate aerobically at 37° C.	Colonies 1-2 mm. in diameter after 24 hours' incubation. Low convex, slightly crumpled edge and matt surface. Some differentiation into outer gray and inner darker zones. Moved as a whole when touched and had a "cold margarine" consistency. Usually surrounded by a small zone of hemolysis. Colonies 2-5 mm. in diameter after 48 hours' incubation. Flattened colony with raised central papilla, some differentiation into inner darker and outer paler zones. Irregular edge	Colonies 0.5-1 mm. in diameter after 24 hours' incubation. Low convex, entire edge and matt surface. Little differentiation into inner and outer zones. Matt, black colour, moving as a whole when touched and has "cold margarine" consistency. Most strains surrounded by small zone of hemolysis. Colonies 1-4 mm. in diameter after 48 hours' incubation. Flattened colony with black centre and lighter periphery. Irregular edge which drops sharply to surface of medium	Colonies small, uniformly blackish, low convex with matt surface after 24 hours' incubation. Increased only very slightly in size after a further 24 hours' incubation. Colonies remain round and become more convex and surface presents a rougher appearance than formerly
Growth in heart broth aerobically at 37° C.	Surface pellicle and usually coarse granular sediment after one or two days' incubation. Turbidity may develop throughout broth but it may be late or absent	Organisms grow readily in heart broth, producing turbidity throughout medium, sediment and a surface pellicle in one or two days	Scanty growth in broth; strains examined formed a surface pellicle in two or three days. Some sediment but little turbidity in medium

production. (3) Methyl-red test. (4) Voges-Proskauer reaction. (5) Nitrate reduction. (6) Gelatin liquefaction at 37° C. and on a slope, and in a stab at room temperature. (7) Action on litmus milk. The results are summarised in table II.

### *Animal experiments*

These were performed in guinea-pigs only. Intradermal tests were carried out with all the starch-fermenting, gelatin-liquefying strains, one strain of *C. diphtheriæ* var. *gravis*, one of *C. diphtheriæ* var. *intermedius*, a known avirulent strain of *C. diphtheriæ* var. *mitis* and five strains of other corynebacteria.

The five other strains of *Corynebacterium* chosen for intradermal injection were one each of *C. ovis*, *C. murium* and *C. pyogenes*, all from the National Collection of Type Cultures, and two biochemically active strains (J/A 8285 and J/G 1314) isolated here.

One strain of starch-fermenting gelatin-liquefying *Corynebacterium* was also investigated by subcutaneous injection into guinea-pigs.

*Intradermal tests.* (Römer, 1909; Zingher and Soletsky, 1915), The growth from an 18-hour Loeffler slope was suspended in approximately 2 ml. of Hartley's broth by adding the broth to the tube containing the slope and scraping the growth into the broth with a loop, broth being used as a suspending fluid in view of the recommendations of Fraser and MacNabb (1937) and Holt and Wright (1940). Of this suspension 0.2 ml. was injected intradermally into the shaved abdomen of each of two guinea-pigs weighing 200-300 g. each. Immediately after the injection one guinea-pig received 10 units of diphtheria antitoxin subcutaneously (Mair, 1930); the other received 1000 units.

Suspensions of *C. diphtheriæ* var. *gravis*, of *C. diphtheriæ* var. *intermedius* and of a known avirulent strain of *C. diphtheriæ* var. *mitis* were also injected intradermally into each guinea-pig. Suspensions of other corynebacteria were also occasionally injected; seven or eight injections were made into each guinea-pig. The animals were inspected on the three days after the injections and at intervals during the next ten days. The results of the intradermal tests are summarised in table III.

*Subcutaneous tests.* 0.5 ml. of a broth suspension of the growth from an 18-hour Loeffler slope culture of the organism to be tested was injected subcutaneously into the right thigh of a guinea-pig weighing 200-300 g. The antitoxin was injected into the left thigh, also subcutaneously (Mair, 1930).

One strain of starch-fermenting gelatin-liquefying *Corynebacterium* was injected into four guinea-pigs. One had received no antitoxin, the other three had received 500, 1000 and 10,000 units respectively of diphtheria antitoxin 24 hours before the organisms were injected. The unprotected guinea-pig died within 24 hours and at post-mortem

TABLE II—Biochemical reactions of starch-fermenting, gelatin-liquefying strains compared with those of other corynebacteria

Strain	Number tested	Glucose	Maltose	Sucrose	Starch	Dextrin	Glycerol	Mannitol	Galactose	Trehalose	Indole	Methyl-red	Voges-Proskauer	Nitrate reduction	Gelatin liquefaction			Litmus milk
															Slope 22° C.	Slab 22° C.	Slab 37° C.	
Starch-fermenting gelatin-liquefying corynebacteria	26	AC (1-2)	24 AC (1-4) 2A (2)	23-2A (1-4-20) 1 AC (1-4)	AC (1-4)	AC (1-6) 8a or A (8-19) 4no or AC (17-21)	14-8a or A (8-19) 4no or AC (17-21)	—	20 Ao or AC (1-19) 6A (1-3)	AC (6-10)	—	22-25	—	—	+	—	—	Unchanged or slightly alkaline
<i>C. diptheria</i> var. <i>gravis</i>	5	AC (1)	AC (1)	—	AC (1)	AC (1-4) 3 AC (2-8)	AC (17-21) (3-6)	—	AC (1)	—	—	4-1	—	+	—	—	—	Unchanged
<i>C. diptheria</i> var. <i>intermedius</i>	5	AC (1)	AC (1-2)	—	—	2-AC (2-7) 2 AC (1-2)	AC (10-13)	—	AC (1-3)	—	—	—	—	+	—	—	—	Unchanged
<i>C. diptheria</i> var. <i>mitis</i>	5	AC (1)	AC (1-6)	—	—	2-AC (2-7) 2 AC (1-2)	2-3 AC (6-10) 2 AC (5-6)	—	AC (1)	—	—	1-4	—	1± 2	—	—	—	Unchanged or slightly acid
<i>C. ovis</i>	4	AC (1-2)	AC (1-10)	1-1 AC (10) 2A (13)	—	2 AC (1-2) 2A (4-18) Ao (6)	2-3 AC (6-10) 2 AC (5-6) 2a (10)	—	AC (1-2)	—	—	—	—	1± 3	—	—	—	Unchanged or slightly alkaline
<i>C. pyogenes</i>	1	Ao (2)	AC (17)	2A (13)	Ao (10)	—	—	—	Ao (6)	—	—	—	—	+	—	—	—	Acid clot
<i>C. murium</i>	1	A (12)	A (12)	no (13)	—	—	—	—	—	—	—	—	—	+	—	—	—	Alkaline
<i>C. xerosis</i>	1	AC (1)	A (6)	AC (1-4)	—	—	—	—	AC (4)	—	—	—	—	+	—	—	—	Slightly alkaline
<i>C. equi</i>	1	—	—	—	—	—	—	—	—	—	—	—	—	+	—	—	—	Slightly acid
<i>C. hojmannii</i> J/A 8285	1	AC (12)	AC (12)	AC (10)	—	A (7)	A (19)	AC (9)	AC (15)	AC (15)	—	—	—	+	—	—	—	Alkaline
J/A 1314	1	AC (4)	AC (10)	Ao (10)	—	—	—	—	—	—	—	—	—	+	—	—	—	Transient acidity
J/A 8515	1	AC (6)	—	AC (5)	—	—	—	—	—	—	—	—	—	+	—	—	—	Unchanged
J/A 10220	1	AC (2)	—	—	—	—	—	—	AC (4)	—	—	—	—	+	—	—	—	Slightly alkaline
J/A 7096	1	—	—	—	—	—	—	—	—	—	—	—	—	+	—	—	—	Alkaline

AC = acid and clot, no = slight acidity and slight clotting, A = acid, a = slight acidity, Ao = acid and slight clotting, + = reaction positive, — = reaction negative or no fermentation noted, ± = reaction weakly positive.

Figures in parenthesis denote number of days required for reaction. Other figures denote number of strains giving that reaction.

TABLE III  
Results of intradermal tests in guinea-pigs

Organism	Number of strains tested	Results in guinea-pigs which received 10 units of diphtheria antitoxin						Results in guinea-pigs which received 1000 units of diphtheria antitoxin					
		Days after injection						Days after injection					
		1st	2nd	3rd	5th	7th	10th	1st	2nd	3rd	5th	7th	10th
Starch-fermenting, gelatin-liquefying corynebacteria	20	+, often slough	++ or +, slough or ulcer	++ or +, ulcer and often pus	+ ulcer or healing ulcer	± or + healing ulcer	healed	++ or +, often slough	++ or +, slough or ulcer	++ or +, ulcer	++ or +, ulcer or healing ulcer	± or +, healing ulcer sometimes pus	healed or ± ulcer or pus
<i>C. diphtheriae</i> var. <i>gravis</i>	1	± or +	+ or ++	+	± or +	— or ±	—	—	—	—	—	—	—
<i>C. diphtheriae</i> var. <i>intermedius</i>	1	± or +	+ or ++	+ or ++	±	—	—	—	—	—	—	—	—
<i>C. diphtheriae</i> var. <i>mitis</i> (avirulent)	1	—	—	—	—	—	—	—	—	—	—	—	—
<i>C. ovis</i>	1	+	+	+	+ u.	+ h.u.	h.	+	+	+	+ pus	± h.u.	h.
<i>C. pyogenes</i>	1	—	—	—	—	—	—	—	—	—	—	—	—
<i>C. murium</i>	1	—	—	—	—	—	—	—	—	—	—	—	—
Diphtheroid J/A 8285	1	—	—	—	—	—	—	—	—	—	—	—	—
Diphtheroid J/G 1314	1	—	—	—	—	—	—	—	—	—	—	—	—

u. = ulcer.

— = no lesion seen.

++ = lesion 10-15 mm. diameter.

h.u. = healing ulcer.

± = lesion less than 5 mm. diameter.

+++ = lesion more than 15 mm. diameter.

h. = healed.

+ = lesion 5-10 mm. diameter.

there was some free fluid in the peritoneal and pericardial cavities. The suprarenals were engorged and hæmorrhagic.

Of the guinea-pigs which received antitoxin two survived for the month they were kept under observation. The third, which had received 10,000 units, developed a large ulcer at the site of inoculation and subsequently died of a presumably intercurrent infection. No change was seen in the suprarenals *post mortem*. No blood culture was made from this guinea-pig.

Controls were injected with 0.5 ml. of a suspension of *C. diphtheriæ* var. *gravis*. The unprotected guinea-pig died within 24 hours with the signs typical of diphtheria. The protected guinea-pig received 500 units of antitoxin 24 hours before 0.5 ml. of *gravis* suspension, and it remained alive and apparently well.

### DISCUSSION

It is apparent from the foregoing description of these starch-fermenting gelatin-liquefying strains, that the organisms with which they are most likely to be confused are *C. diphtheriæ* var. *gravis* and *C. ovis*. Morphology and cultural characteristics are of little help in distinguishing these organisms from each other, though on blood-agar plates the colonies of the starch-fermenting gelatin-liquefying strains and of *C. ovis* are yellow and more opaque than those of *C. diphtheriæ*. The colonies on blood-tellurite-agar are also rather more uniform in colour than those of *C. diphtheriæ*.

The distinction of these strains from *C. diphtheriæ*, *C. ovis* and the other corynebacteria examined thus depends mainly on biochemical reactions and the results of animal experiments. The biochemical tests which help to distinguish the starch-fermenting gelatin-liquefying strains from *C. diphtheriæ* var. *gravis* and *C. ovis* are summarised in table IV.

TABLE IV

*Biochemical reactions which help to distinguish the starch-fermenting, gelatin-liquefying strains from C. ovis and C. diphtheriæ* var. *gravis*

	No. tested	No. positive	Glucose	Maltose	Sucrose	Starch	Dextrin	Glycerol	Trehalose	Gelatin liquefaction	Nitrate reduction at 5 days
<i>C. diphtheriæ</i> var. <i>gravis</i>	5	in 2 days in 21 days	5 5	5 5	0 0	5 5	3 5	0 5	0 0	0 0	5
Starch-fermenting, gelatin-liquefying corynebacteria	26	in 2 days in 21 days	26 26	23 24	0 1	23 26	25 26	0 3	0 26	0 26	0
<i>C. ovis</i>	4	in 2 days in 21 days	4 4	2 4	0 1	0 0	2 2	0 2	0 0	0 1	1

In this table only strains which produced acid and clot in a sugar in the stated period are regarded as giving a positive reaction in that sugar. Certain other strains produced acid only in some of the sugars (see table II).



The chief points which distinguish the starch-fermenting gelatin-liquefying strains are: (1) their late fermentation of trehalose; (2) their failure to reduce nitrates to nitrites—this test is not by itself of any great differential significance since many other corynebacteria fail to reduce nitrates (see table II and also Brooks and Hueker 1944); and (3) their liquefaction of a gelatin slope at room temperature.

### *Animal experiments*

Intradermal injection of guinea-pigs shows that whereas the local reactions caused by virulent strains of *C. diphtheriae* can be completely neutralised by the injection of 1000 units of diphtheria antitoxin, those produced by the starch-fermenting gelatin-liquefying strains cannot be neutralised by such a dose. Since this amount of antitoxin had little effect on the size or nature of the lesion, it seems that some at least of the local reaction caused by these organisms may be attributed to the ovis-like fraction of the toxin which they elaborate (Petrie and McClean, 1934).

When a suspension of a starch-fermenting gelatin-liquefying *Corynebacterium* is injected subcutaneously, however, diphtheria antitoxin may protect a control guinea-pig from death, as was found with Mair's strain (Mair, 1928) and with the strain which I injected subcutaneously. With other strains, however, 10,000 units of diphtheria antitoxin will not protect from death (Wright, 1942; Robinson, 1944, personal communication).

If virulence tests are performed by the intradermal and subcutaneous routes it may thus happen that the subcutaneous test gives a result similar to that given by a virulent strain of *C. diphtheriae*, while the intradermal test shows that atypical lesions are produced in both test and control guinea-pigs.

So few starch-fermenting gelatin-liquefying strains have thus far been reported that their significance is still doubtful. Some of these organisms elaborate a toxin which is neutralisable, in part at least, by diphtheria antitoxin, and it would seem wise, therefore, to regard the infections they cause with considerable suspicion. Some of the strains have been isolated from cases regarded as clinical diphtheria, and some of the patients have been seriously ill.

Whatever the significance of such an organism may be in an individual case, the only record of outbreaks due to gelatin-liquefying strains is that of Gilbert and Stewart (1928-29), who record three outbreaks of clinical diphtheria associated with *C. ulcerans*. The outbreaks were spread over a period of four years, and the presence of *C. diphtheriae* could not be definitely excluded from any of them.

## SUMMARY

Twenty-six strains of starch-fermenting gelatin-liquefying corynebacteria, twenty-four of them from the human nose or throat, have been examined.

These organisms are in many ways similar to both *C. diphtheriæ* and *C. ovis*. They can be distinguished from these organisms and the other strains of corynebacteria examined by: (1) their late fermentation of trehalose in conjunction with their earlier fermentation of glucose, maltose, starch, dextrin and galactose; (2) their liquefaction of a gelatin slope at room temperature; (3) their failure to reduce nitrates to nitrites; and (4) their behaviour on intradermal injection into guinea-pigs.

In guinea-pigs all the strains examined produced cedema and ulceration, and usually pus, whether the animal received 10 or 1000 units of diphtheria antitoxin. On subcutaneous injection of these strains diphtheria antitoxin may or may not protect a guinea-pig from death.

So few strains have been reported that no certain conclusion about their epidemiological importance may yet be drawn.

I am indebted to Dr R. E. Jones of Hereford, Dr B. Moore of Exeter and Dr A. J. H. Tomlinson of the Central Public Health Laboratory, Colindale, who kindly sent me strains for investigation.

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### Appendix

The fermentation reactions were studied in Hiss's serum water containing 1 per cent. of the carbohydrate to be tested and Andrade's indicator.

The base for the sugars is prepared in bulk in the following manner:—

Peptone . . . .	7.0 g.
Na <sub>2</sub> HPO <sub>4</sub> . . . .	1.4 g.
Distilled water . . . .	1400 ml.

Steam for 15 minutes and filter through paper. Adjust pH to 7.4 and add 250 ml. horse serum. Steam for 20 minutes, and add 11 ml. Andrade's indicator. The pH should now be 7.6-7.8. Autoclave at 10 lb. for 10 min.

To make up individual sugars the base is put into 6" ×  $\frac{1}{2}$ " test-tubes in 3 ml. quantities, the sugar is made up as a 10 per cent. solution and 0.3 ml. added to each 3-ml. tube of serum water. The sugars are then sterilised by steaming for twenty minutes on each of three successive days.

In this investigation the tubes were inspected and the results recorded each day for three weeks, and then discarded.

The presence of indole was tested for in five-day cultures in 1 per cent. peptone water, using Bohme's solution A after extraction with ether.

The methyl-red test was performed on five-day cultures in glucose-phosphate medium by adding five drops of a 0.04 per cent. solution of methyl red.

The Voges-Proskauer test was performed on five-day cultures in glucose phosphate medium by O'Meara's (1931) modification of the Voges-Proskauer test.

The test for nitrate reduction was performed by the Griess-Ilosva method (Wilson and Miles, 1946) on a culture grown for five days at 37° C. in broth containing 0.1 per cent. potassium nitrate.

Gelatin liquefaction was tested on 15 per cent. gelatin dissolved in nutrient broth. Slopes and stabs kept at room temperature were not discarded as negative until after four weeks. Those incubated at 37° C. were read after one week.

The litmus milk was observed over a period of three weeks and any changes were recorded on the day on which they were noticed.

All these media were inoculated with a loopful of an 18-hour culture on a Loeffler's serum slope.

## FIBRINOLYSIN AND THE FLUIDITY OF THE BLOOD *POST MORTEM*

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MORGAGNI (1769) seems to have been the first to record an autopsy where the blood was entirely fluid. He described the case of a man he saw in 1725 who died shortly after being stabbed through the heart, and suggested that the fluidity of the blood was due to its dilution by the quantity of drink he had taken just before he was stabbed. Later, Hunter (1794) wrote: "In many modes of destroying life the blood is deprived of its power of coagulation, as happens in sudden death produced by many kinds of fits, by anger, electricity or lightning; or by a blow on the stomach etc. In these cases we find the blood, after death, not only in as fluid a state as in the living vessels, but it does not even coagulate when taken out of them". Subsequent investigations usually centred on the forensic aspects of the problem, and conclusions as to the nature of the phenomenon, and even as to its existence, were contradictory until Morawitz (1906) showed that the fluid blood was free from fibrinogen and sometimes contained a fibrinolysin. Fidon, Gautier and Martin (1908) and Oki (1934) confirmed the occurrence of fibrinolysis in experimentally asphyxiated dogs. Recently the phenomenon was turned to practical use in the preparation of cadaver blood for transfusion (Yudin, 1936).

Lenggenhager (1938) seems to have been the first to ask the more general question why the blood in a cadaver is never found completely coagulated. Normally, blood is spontaneously coagulable and after death the vessels should be filled either with a cast of coagulum or with fluid yet spontaneously coagulable blood. However Virchow (1871) noted that the capillary blood of the cadaver is always fluid and incoagulable, and the blood in the veins of the limbs, although more often fluid than not at the ordinary autopsy, is only exceptionally still coagulable.

A reinvestigation was therefore made of the conditions which determine the fluidity of the blood at autopsy, and of the properties of the fibrinolysin to be found there.

### METHODS

Routine autopsies were used for observations as opportunity offered. No consecutive series was attempted and examples of dramatic and sudden death

were investigated when possible. Blood was obtained either by syringe and needle or by catching it in a container as it flowed out of an incision made in the vessel. The red cells were centrifuged down and the supernatant "serum" used for observations on fibrinolysin.

#### *Detection of fibrinolysin*

The method of Macfarlane (1937) was used with some modification. Citrated "blood-bank" plasma (0.5 c.c.) was used as a source of fibrinogen and diluted with normal saline, borate buffer pH 7.6 or phosphate buffer pH 7.4 in a stoppered tube. The standard volume (0.25 c.c.) of "serum" to be tested was then added and lastly 0.5 c.c. of 0.1 M calcium chloride or of a thrombin solution. The final volume was adjusted to 10 c.c. by varying the original volume of diluent. After being inverted to mix, the tube was placed in the 37° C. air incubator and observed at intervals. A white fibrin clot formed in a few minutes and later disappeared, leaving a solution identical in appearance with that seen before the clot had begun to form. The activity of the lysin was measured by the interval between mixing the reagents and the disappearance of the clot. Mechanical disturbance of the clot was avoided, as the partially lysed clot could easily be converted into shreds of fibrous material by incautious shaking, thus altering the end-point.

The dilution of plasma was about 1 in 30, the fibrinogen concentration about 10 mg. per 100 c.c. and the dilution of serum under test 1 in 40.

In the experiments on the chemical properties of the lysin all reagents were brought to 37° C. before mixing. The pH was determined by the Morton glass electrode with a potentiometer. Fibrinogen was estimated by nephelometry after conversion to fibrin.

#### RESULTS

At autopsy the blood in the heart and great vessels may be largely clotted or completely fluid, or may show intermediate degrees of coagulation. In table I are collected 61 cases where the blood in the great vessels was either solidly clotted or completely or almost completely fluid. The cases are grouped according to the cause of death. It will be seen that in cases of death from infection and cachexia the blood was clotted, whereas in cases of "sudden" death the blood was fluid. The more "sudden" the death, with exceptions to be discussed later, the more likely was the blood to be found completely fluid.

In none of the cases in table I did samples of blood coagulate spontaneously *in vitro*. Thromboplastin (Russell viper venom alone or with added lecithin) and thrombin (Maw, or clotting globulin Lederle) in varying amounts failed to clot the samples, and every attempt to salt out fibrinogen was unsuccessful. Heating the supernatant "serum" to 55° C. produced a precipitate only occasionally, and this never had the flocculent character of heat-denatured fibrinogen in normal plasma. It was concluded that the blood at autopsy lacked fibrinogen and was thus not spontaneously coagulable.

When the vessel from which the blood was obtained contained a visible solid clot there was a satisfactory explanation of the loss of fibrinogen. When, however, there was no clot in the vessel the absence of fibrinogen had still to be explained.

TABLE I

*Cause of death and fluidity of the blood in 61 post-mortems*

	Blood quite fluid	Small clots in heart with fluid blood in limbs	Abundant clot in heart and also in limb vessels
<b>Cranial lesions</b>			
Gunshot wound . . .	2 (suicide) 1 (accident) 1 (accident)	...	1 (suicide)
Cerebral tumour . . .	2 (each shortly after needling)	3	2 (1 X-ray necrosis of brain) (1 pneumonia)
Cerebral abscess . . .	1 (subdural abscess with sterile pulmonary abscess following penicillin)	...	3 (1 with meningitis) (1 with sinusitis and subdural abscess) (1 with empyema)
Hæmorrhage . . .	4 (3 proved congenital aneurysms)	3 (1 leukaemia)	...
Miscellaneous . . .	...	1 (chronic encephalomalacia) 1 (cerebral laceration with multiple injuries)	1 (extradural hæmatoma and pneumonia)
<b>Circulatory lesions</b>			
Hæmorrhage . . .	2 (1 duodenal ulcer with eroded artery) (1 gunshot wound of femoral artery)	...	...
Pulmonary embolism . .	1 (with inhaled vomit)	...	1 (with pneumonia)
Myocardial infarction . .	2 (rupture of ventricle)	1	...
"Heart failure" . . .	1 (chronic bronchitis with old myocardial infarction)	1 (Harvey's syndrome) 1 (Klippel-Feil syndrome)	1 (old rheumatic heart with thrombosis in pulmonary artery)
<b>Anoxic deaths</b>			
While anæsthetised . . .	1 (operation for prolapse)	1 (operation on crushed finger)	...
Drowning . . .	3 (2 inhaled vomit)	...	...
Bronchial obstruction . .	2 (1 by pus from bronchiectatic cavities during needling of cerebral abscess) (1 by blood from tracheotomy above site of tracheal obstruction)	1 (hæmoptysis in pulmonary tuberculosis)	...
Tetanus . . .	1	1	...
<b>Poisoning</b>			
	4 (1 cyanide) (1 carbon monoxide) (1 insulin coma in schizophrenia) (1 lysol)	...	1 (carbon monoxide)
<b>Infection and cachexia</b>			
Infection . . .	...	...	2 (multiple lung abscess) (bronchiectasis) (empyema and otitis media) 5 (pneumonia) (2 uræmia) (1 strangulated hernia) (1 bilateral senile gangrene) (1 cellulitis following lithotomy)
Malignant disease . . .	...	...	3 (carcinomatosis)

No evidence was found of an agent that destroyed fibrinogen, but a lysin for fibrin was often demonstrated. Indeed it was found in every case in table I where the blood was completely fluid.

In intermediate cases, not recorded in table I, where the blood was partly fluid and partly clotted, it was noticed that the fluid blood was found in the vessels of the limbs, the clotted blood in the heart. Results of fibrinolysin tests in these cases are given in table II and

TABLE II

*Fluidity of the blood and fibrinolysin level in autopsies  
where both heart and limb blood were examined*

Degree of fibrinolysis	Blood firmly and abundantly clotted			Blood completely fluid		
	Origin of blood sample			Origin of blood sample		
	Heart	Arm	Leg	Heart	Arm	Leg
—	10	1	2	2	1	2
+	2	1	0	1	0	0
++	1	1	1	0	2	5
+++	0	0	0	3	13	13

— = no lysis.

+ = lysis overnight.

++ = lysis 2-6 hours.

+++ = lysis 0-2 hours.

show that in vessels where the blood was completely fluid an active lysin was usually present, whereas in vessels where the blood was clotted, lytic activity was usually absent and never marked. Further, the results show that lysin in limb blood was more active than in heart blood, even when the blood in each was completely fluid.

#### *In-vitro behaviour of the fibrinolysin*

Fibrinolysin added to blood or plasma after a clot had formed was not effective. Lytic activity was shown only if the lysin was present while the clot was being formed. The lysin is adsorbed on the clot and later released into solution when the fibrin is lysed. Thus a given concentration of fibrinolysin is more likely to liquefy a clot the slower the process of coagulation and the smaller the amount of fibrin formed at any one time. Fibrinolysin did not appear to affect fibrinogen or the process of coagulation. It was possible, however, for fibrinolysis to be so active that fibrin was destroyed as rapidly as it was formed, so that no clot was ever visible.

These observations were all made using the standard test system in which the inhibitory effect of serum was minimised by dilution (Macfarlane). There is a considerable reduction in lysin concentration and an increase in the rate of coagulation in the test system compared

with intravascular whole blood. Both these factors tend to compensate for the lack in the in-vitro system of the inhibitory action of neat serum, and to make this test not as dissimilar as might have been expected from the behaviour of whole blood within the vessels.

A direct comparison of the in-vitro and intravascular behaviour of whole blood may be made by examining samples of whole blood obtained by cardiac puncture after death. Lysis of clots formed from such specimens often failed to occur, even though a later autopsy demonstrated the complete fluidity of the heart blood, and serum obtained then was strongly active in the fibrinolytic test system, using diluted plasma. This was presumably because the rate of coagulation was more rapid *in vitro*, making fibrinolysis less effective, as already explained. Complete lysis of whole-blood clots obtained by cardiac puncture was seen, but only when natural in-mortuo lysis was extremely rapid and active as judged by the short interval between death and complete loss of fibrinogen (table III).

TABLE III

*Fluidity of blood obtained by cardiac puncture in cases of sudden death*

	Interval till death (hours)	Time after death when sample of heart blood was obtained (hours)	
		Blood clotted in <i>vitro</i>	Blood remained fluid in <i>vitro</i>
Traumatic rupture of liver . . .	$\frac{1}{2}$	$\frac{1}{2}$ *	$1\frac{1}{2}$
Gunshot wound of head (accident)	$\frac{1}{2}$	...	$2\frac{1}{2}$
Hyperpyrexia (heat stroke) . .	3	$1\frac{1}{2}$ *	$2\frac{3}{4}$
Poliomyelitis (in iron lung) . .	?	...	4
Drowned . . . . .	?	...	5
Fractured spine (C. 5) . . . .	48	3	6
Tetanus . . . . .	?	7	10
		(very feeble clot)	

\* These clots lysed spontaneously in one hour at 37° C.

### *The source of the fibrinolysin*

After clamping the veins, samples of blood were obtained from different regions of the body in order to see if there was any regional difference in lytic activity. There was a centripetal decrease in activity, confirming the findings in table II, where limb blood was more active than heart blood. Portal-vein blood was more like heart blood than limb blood in its earlier in-mortuo coagulation, in the more frequent presence of large clots, and in the less frequently occurring and less powerful lysis. No systematic difference was found between the left and right sides of the heart.

Thus there was no evidence that the fibrinolysin was liberated from any particular organ. The approximate inverse relationship between the activity of the lysis and the diameter of the vessel from



which the sample of blood was obtained suggested that the lysin might be the product of the vascular endothelium. The other possible source seemed to be the leucocytes of the blood.

A length of femoral vein was ligated *in situ* shortly after death and left till next day. The lytic activity of the "serum" was compared with that of the "serum" obtained at the time of ligation from the corresponding part of the other femoral vein and preserved in the refrigerator. The activity in each sample was identical, showing that even if the endothelium was the source of the lysin it yielded no further lysin once death had occurred and the blood had become fluid and incoagulable.

Fibrinolytic activity was also found in the pericardial fluid of a case where the blood was completely fluid and actively lytic. The fluid in the pericardium was as active as the "serum" from the heart, and presumably its activity must have been derived from the pericardial endothelium. The absence of fibrin and the very low fibrinogen levels found in hæmothorax fluid by Sellors (1945) support the view that endothelium is a source of fibrinolysin.

Nevertheless autopsies occurred where clotted blood was found in the body cavities and fluid blood in the blood vessels and heart, *e.g.* a case of traumatic rupture of the liver with a large unretracted blood clot occupying the site of the rupture and a case of ruptured myocardial infarction with pericardial clot. In cases such as these the stimulus to fibrinolysin production is not likely to have preceded the leaking of blood into the endothelial-lined cavities. The blood in the pericardial or peritoneal cavity will have clotted rapidly because of the thromboplastin added to it from the torn tissue surfaces past which it has flowed. Rapid coagulation will protect the clot from any fibrinolysin subsequently entering the blood stream or being given off by the endothelial lining of the cavity, since to be effective the lysin must be present while the clot is being formed.

*The conditions under which fluid but spontaneously  
coagulable blood is found at autopsy*

In some cases in which the autopsy happened to be carried out at the appropriate time, the blood in the heart was found to be firmly clotted, whereas that in the veins of the arms and legs was fluid but coagulable. The slower onset of spontaneous clotting in the great veins of the limbs as compared with the heart showed that there was a regional difference in the rate of spontaneous intravascular coagulation as well as in fibrinolytic activity, but in the opposite sense. These two factors, reinforcing each other, accounted for the greater frequency of fluid and incoagulable blood in limb vessels as compared with the heart.

Fluid and coagulable blood was always found whatever the cause of death when the autopsy was carried out within an hour or so of

death. In order to discover how quickly the blood could become incoagulable and thus how quickly fibrinolysin could act within the vessels, serial cardiac punctures were carried out on a few selected cadavers in which the blood would be expected to be fluid and incoagulable at a later autopsy. The results (table III) show that in cases of "sudden" death the blood was spontaneously coagulable only during a brief period after death and then became completely free from fibrinogen. To confirm that the heart contained no clot and that the sample of blood removed by cardiac puncture was not merely the defibrinated blood expressed from a clot in the heart cavities, the autopsy was carried out in each case as soon as it was clear that the heart blood was no longer coagulable.

On three occasions spontaneously coagulable fluid blood was found more than twenty-four hours after death. In these cases death had been due to a long continued cachectic process, *e.g.* pylophlebitis. The mode of death therefore affected the coagulability of the blood after death, as well as the activity of fibrinolysin.

#### *Exceptions to the appearance of fibrinolysin after sudden death*

In cases of "sudden" death in previously healthy persons the blood was usually fluid and showed fibrinolytic activity. Cases of death with multiple traumatic injuries, however, did not show fluid blood or fibrinolysis to the degree which might have been expected. In some, early pneumonia may have been the significant factor, in the same way that infection appeared to prevent the occurrence of fluid blood in death from cranial lesions (table I). In four cases there seemed to be an association of fat embolism with partial failure of fibrinolysin production, *e.g.* the heart was full of clot and the expressed serum devoid of activity, while the arm and leg veins contained fluid blood, the serum from which was actively lytic. Marrow fat is markedly thromboplastic and rapid and complete coagulation may have prevented the dissolution of the clot by fibrinolysin (*vide supra*). That this may not be the whole explanation, however, is suggested by the spontaneous coagulability of the blood in one of the cases as long as twenty-four hours after death, which seems to bring fat embolism into the same group as infection and cachexia.

Age had some influence. Infants did not show fluid blood as frequently as adults, confirming the observations of Roll (1918) on death from drowning.

A few more notable exceptions occurred, of which the most striking were a case of accidental carbon monoxide poisoning and a case of suicidal gunshot wound of the head. Each had been drinking heavily all evening before his death, and in each the blood was coagulated and no fibrinolytic activity was detected.

*The properties of cadaver fibrinolysin*

The lysin was non-dialysable. Fat solvents destroyed some of the lytic activity without extracting any lysin. Chloroform was not used. At neutral pH levels the lysin was precipitated by 50 per cent. saturation with ammonium sulphate. It was not precipitated with the euglobulin fraction of serum. Fibrinolytic activity was completely destroyed by pepsin at pH levels where, without pepsin, the lysin was stable. The fibrinolysin thus appeared to be a globulin.

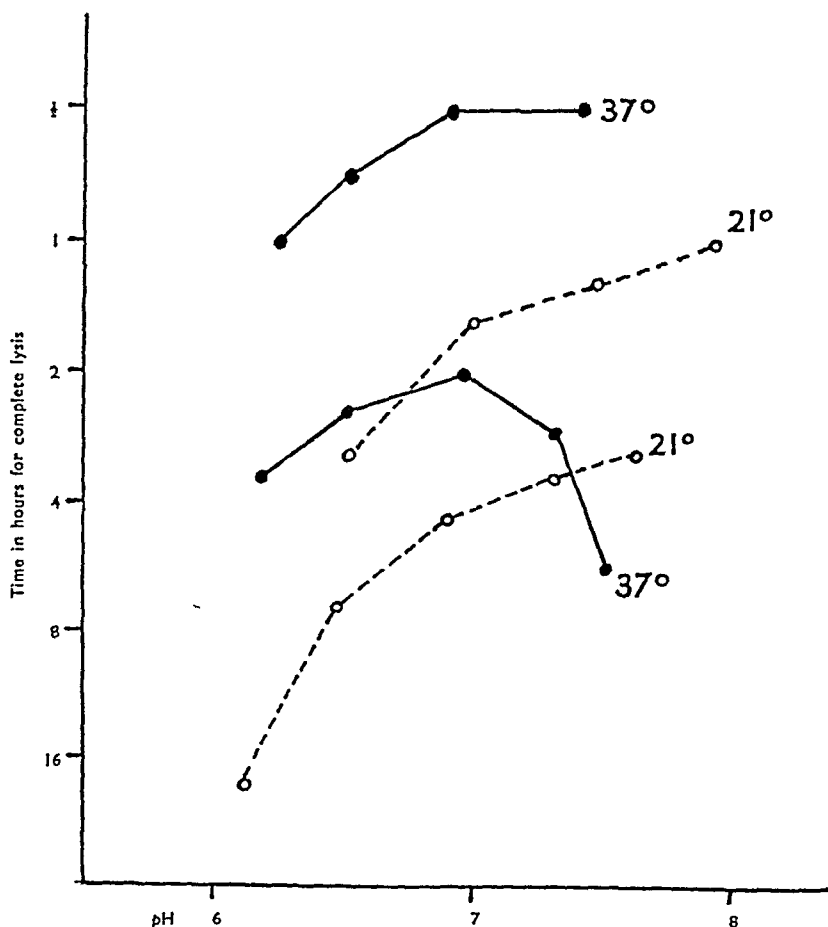


FIG.—Effect of temperature and pH on fibrinolytic activity.

Post-mortem sera retained their fibrinolytic activity in the refrigerator for days and sometimes weeks. At room temperature half the activity was sometimes lost overnight. At higher temperatures stability of the fibrinolysin was shown to depend on pH. At pH 3-4 little activity was lost in one minute at 90° C., while at pH 7.4 there was partial loss of activity at 60° C. for ten minutes and complete inactivation at 70° C.

The optimum pH was on the alkaline side of neutrality, as was also found by Oki, but inactivation also occurred with increasing rapidity the more alkaline the pH. As shown in the figure on p. 420, the optimum pH at 21° C. was the same whether concentration of lysin was high or low. At 37° C. the less active the lysin the more acid the optimum pH, since lysin was inactivated at more alkaline pH levels during the longer incubation period necessitated by the weaker lysin.

No other substrates than fibrin and fibrinogen were examined. A concentration of lysin which was able to lyse a fibrin clot completely in twenty minutes did not appear to affect fibrinogen in oxalated or citrated plasma, even after two hours at 37° C.

There was an approximately linear relation between the logarithm of the concentration of the lysin and the logarithm of the time for complete lysis.

If a solution which had become water-clear after fibrinolysis was kept overnight a white precipitate usually formed.

### *Inhibitors*

Normal serum was increasingly inhibitory in concentrations of 10 per cent. and higher. The standard test system was chosen so that plasma and serum to be tested for activity were each diluted at least twenty times. The inactivity of normal serum is therefore not due to the presence of an excess of serum inhibitor but to the absence of an active lytic factor. This was confirmed in experiments where the proteins of serum were denatured by heat in acid solution. No lytic activity was uncovered in normal sera or in inactive cadaver sera.

Heparin in concentrations which doubled the clotting time had no effect on the time from coagulation to complete lysis, whether or not serum was also added.

Phenyl mercuric nitrate was ineffective even in a concentration of 1 : 10,000 in the standard test system.

### DISCUSSION

Lenggenhager thought that the blood was fluid only when the heart beat outlasted respiratory activity so that "internal asphyxia" and hence acidosis was produced. This was part of his theory that acidosis was the essential feature which made the blood fluid, a theory which supposed that after death from shock due to immersion in water the blood is not fluid, while in death from true drowning it is, and that in death from ventricular fibrillation the blood is not fluid, while in somewhat protracted death, *e.g.* from coronary occlusion, the blood was fluid because respiration stopped before the heart. It was a theory difficult also to reconcile with the firm abundant

clot to be found *post mortem* in such instances of severe acidosis as pneumonia.

The observations here recorded confirm the earlier findings (Falk, 1874; Morawitz, 1906), and show that samples of completely fluid and incoagulable blood are always free from fibrinogen and in at least 90 per cent. of cases contain a fibrinolysin. Fibrinogenolysis, for which evidence was obtained by Morawitz, was not found. The inverse relationship between the activity of the lysin and the presence of clots (table II) provides additional support for the view that a fibrinolysin is the factor which makes post-mortem blood incoagulable.

No direct observations are available to show that the blood must clot *in mortuo* before it can become fluid and incoagulable, because opening a blood vessel to direct inspection completely alters the natural course of events. The blood may become incoagulable in  $1\frac{1}{2}$  hours (table III). Nevertheless intravascular coagulation is likely to take place more slowly than in a glass vessel, and as shown earlier the slower the coagulation process the greater the apparent activity of fibrinolysin. Thus fibrinolysin intravascularly may destroy fibrin as rapidly as it is formed, even though, *in vitro*, the same blood would form a solid clot which did not lyse even at 37° C. Since there is appreciable solubility of fibrin in aqueous solution (Jaques, 1938) no tangible clot may ever form, even though all the fibrinogen is converted into fibrin. If the fibrin is never precipitated in the form of fibrils the platelets will not be removed in the meshes of a clot. Thus the finding of platelets in cadaver blood (Lenggenhager) is not proof that the blood has never clotted.

In any attempt to account for the state of the blood at autopsy, the relation between rate of intravascular coagulation after death and fibrinolysin concentration seems decisive. The less active the fibrinolysin and the faster the rate of coagulation, the more likely is the blood to be found clotted, as already explained. Conversely, since limb blood clots more slowly and contains more fibrinolysin than heart blood, there is a greater frequency of incoagulable blood in limb veins than in the heart, and, by extrapolation, capillary blood would be expected to be fluid even more often, as was in fact found by Virchow. If endothelium is the source of fibrinolysin, there is a simple anatomical explanation for the increase in fibrinolytic activity with decrease in the size of that vessel. The factors controlling the rate of intravascular coagulation have not been investigated.

Besides these differences in site, differences in the mode of death also affect the relative activity of these two factors. In cases of sudden death the blood remains spontaneously coagulable for a short period only, which must mean that the rate of intravascular coagulation is rapid. Visible clot, however, is not found in the vessels, because fibrinolytic activity appears to be sufficiently powerful to lyse the fibrin as it is formed. In death from cachexia the rate of intravascular coagulation is greatly reduced, so that the blood remains spontaneously

coagulable for a much longer period. Fibrinolytic "activity" is relatively still further reduced, however, so that after the period of spontaneous coagulability the blood is found coagulated inside the vessels and does not liquefy on further standing.

*The stimulus to the production of fibrinolysin*

The factor necessary for the occurrence of fluid and incoagulable blood has been supposed to be "suddenness" of death (Vogel, 1926), but the data in table I suggest that this is true only if a special meaning is given to the word "sudden". At one extreme it must cover cases where death was almost instantaneous, as in rupture of the heart and cyanide poisoning; at the other, cases where the dying process lasted for many hours (insulin coma, cerebral hæmorrhage) or even days, as in a case of duodenal ulcer with eroded artery which was repeatedly transfused during three days of hæmorrhage. On the other hand, when fibrinolysin did not appear and the blood was coagulated, dying was usually a slow process, but not always, fat embolism being a notable exception. The rate of dying therefore may be less important than some other factor.

Fibrinolysin appeared in the blood after death in persons who were healthy before the "accident" that caused their death. The observations of Macfarlane and his colleagues (Macfarlane, 1937; Macfarlane and Biggs, 1946; Macfarlane and Pilling, 1946; Biggs, Macfarlane and Pilling, 1947) showed that certain stimuli cause the appearance of fibrinolysin in the bloodstream of living healthy individuals, though in much smaller concentrations than in the dead. Death itself therefore is not likely to be the stimulus to fibrinolysin production, but rather whatever it was that led to death. At the same time, the stimuli to the production of fibrinolysin were very diverse (table I), and the common factor can only be the dying process. Many if not all of the dead who showed fibrinolytic activity could have been described as suffering from "shock" or "collapse" while still alive, and the appearance of fibrinolysin in the blood may be a common feature of the shocked state, a state which has so far defied analysis except in so far as hæmorrhage, loss of tissue fluid, pain and peripheral circulatory failure have been abstracted from it. Macfarlane and Biggs suggested that fibrinolysin production might be part of the first phase of Selye's alarm reaction, and indeed some such non-specific "general" reaction to injury, in the widest sense, seems to be the stimulus for fibrinolysin production.

The failure of fibrinolysin to appear in deaths from infection and cachexia, as also noted by Skundina and Rusakov (quoted by Yudin), would thus appear to be due to the failure of one of the body's reactions to injury. Where fibrinolysin did appear after death from infection, as in tetanus and cerebral abscess (table I), death was due to the local and mechanical effects of the infection, not to any generalised

clot to be found *post mortem* in such instances of severe acidosis as pneumonia.

The observations here recorded confirm the earlier findings (Falk, 1874; Morawitz, 1906), and show that samples of completely fluid and incoagulable blood are always free from fibrinogen and in at least 90 per cent. of cases contain a fibrinolysin. Fibrinogenolysis, for which evidence was obtained by Morawitz, was not found. The inverse relationship between the activity of the lysin and the presence of clots (table II) provides additional support for the view that a fibrinolysin is the factor which makes post-mortem blood incoagulable.

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in the fibrinolytic test system. Incubation of the pancreatic enzymes with serum did not modify their activities and it seemed clear that they differed fundamentally from cadaver fibrinolysin, which was also free from thromboplastic activity.

Plasminogen is a globulin found in normal serum and activated by chloroform or streptococcal culture filtrates to form plasmin, a proteolytic enzyme with properties which distinguish it from trypsin (Christensen and MacLeod). Unlike cadaver fibrinolysin, however, plasminogen is a euglobulin, and plasmin is unstable at acid  $pH$  levels and proteolyses fibrinogen at the same rate as fibrin (Christensen, 1945). Thus there seem to be at least two distinct serum proteinases.

Skin proteinase is the first proved tissue proteinase with an optimum  $pH$  on the alkaline side of neutrality and is distinguishable from the pancreatic enzymes by the specificity of its action on synthetic polypeptides (Beloff and Peters). Human skin removed under general anaesthesia was extracted by the method of Beloff and Peters but provided unsatisfactory material for the fibrinolytic test system, since the extracts were markedly antithrombic. The extracts did sometimes show weak fibrinolytic activity, however. What is perhaps more significant is that the presence of activity in the extracts seemed to be related to the illness of the subject from whom the skin was removed, in the same way that the presence of fibrinolysin in a cadaver was determined by the mode of death (*vide supra*). The idea of the similarity of cadaver fibrinolysin and skin proteinase, however, may be merely the result of ignorance of their other properties.

The spontaneously active fibrinolysin described by Macfarlane and his colleagues (*op. cit.*) has not been characterised chemically at all, and it seems premature to assume that it is identical with plasmin. The experimental data of Macfarlane and Pilling are compatible with the alternative explanation that the spontaneously active fibrinolysin was cadaver fibrinolysin produced by the living subject. That this is possible is suggested by the observations here reported that the presence of spontaneously active fibrinolysin in patients post-operatively depends on the nature of the lesion which necessitated operation, just as the occurrence of fibrinolysin in the cadaver depends on the cause of death.

#### *The possible significance of fibrinolysin production during life*

Apart from its appearance as part of the body's reaction to injury, fibrinolysin may have a physiological role, especially if its source is the endothelium of the vascular channels. Blood, if left undisturbed, will always clot eventually, though the interval before clotting begins depends very greatly on the physical properties of the internal surface of the container. However much the physical properties of the vascular endothelium make the endothelial surface anticoagulatory, some deposition of fibrin would be expected. Certainly there is a



toxæmia. The existence of large clots in the main blood vessels is thus not the normal phenomenon, as would have been expected from the behaviour of blood *in vitro*, but the abnormal phenomenon. Emphasis on the fluidity of the blood as characteristic of any special cause or mechanism of death is probably misplaced, especially in the light of the exceptions discussed earlier.

*The relation of cadaver fibrinolysin to other proteases*

Perhaps the most unexpected property of cadaver fibrinolysin is its failure to act on fibrinogen, a property it shares with the fibrinolysin of human prostatic secretion (Huggins and Neal, 1942). Fibrinogen and fibrin have not been distinguished analytically, and X-ray photographs suggest that their molecular pattern is identical (Bailey, Astbury and Rudall, 1943). Fibrin may therefore be an insoluble form of fibrinogen produced by molecular aggregation. If so, cadaver fibrinolysin may be thought of as a depolymerase, and it should be noted that Skundina and Rusakov (quoted by Yudin) failed to find any increase in residual nitrogen as a result of fibrinolysis. Macfarlane thought that post-operative fibrinolysis was due to an alteration in fibrinogen, but his experimental results can best be interpreted as depending on adsorption of the lysin by the clot while it is forming.

Fibrinolysins have been described in blood or its derivatives by Nolf (1908), Rosenmann (1922), Yudin (1936), Macfarlane (1937), Huggins, Vail and Davis (1943), and in human prostatic secretion by Huggins and Neal (1942). Barnes (1940) reviewed the available evidence on the proteolytic enzymes in leucocytes. Direct comparison of cadaver fibrinolysin with any of these proteases is impossible from lack of information as to their properties. Sufficient evidence is available, however, for comparison with other proteases active on the alkaline side of neutrality and inhibited by serum—trypsin and chymotrypsin from the pancreas, plasmin (Christensen and MacLeod, 1945), and the skin proteinase of Beloff and Peters (1944-45).

Cadaver fibrinolysin shares with the pancreatic enzymes the remarkable property of resistance to heat in acid solutions, and their salt-precipitation limits in neutral and acid solutions are also similar. Crystalline trypsin and chymotrypsin, however, destroyed fibrinogen as rapidly as fibrin, unlike cadaver fibrinolysin, and the concentration-activity curves of the pancreatic enzymes in the standard lytic system were quite different. Cadaver fibrinolysin was active over concentration differences of at least a hundredfold without any interference with the clotting of plasma fibrinogen. With trypsin and chymotrypsin, however, there was only a ten- to twenty-fold difference between the lowest concentration which destroyed the fibrinogen before the plasma had had time to clot and the highest concentration which failed to lyse the clot overnight. Crystalline chymotrypsinogen was inactive

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continual production of fibrinogen by the liver which must involve a continual removal of fibrinogen from the blood stream in some way. Fibrinolysin may therefore be the natural physiological means whereby fibrin deposits are prevented from forming on the vascular endothelium in health. It is noteworthy that infection and cachexia, which favour thrombosis, are conditions in which fibrinolysin production fails.

The treatment of hæmothorax depends very largely on whether the fluid is clotted or not. It seems likely that it is the activity of fibrinolysin which determines the outcome, since Sellors found the fibrinogen content of such fluid considerably less than would have been expected from its content of blood. Fibrinolysin may in fact be one of the factors which determine the course of any fibrinous inflammatory process.

Lastly, if Duguid's (1948) finding of fibrin thrombi on the aortic intima is confirmed and if his interpretation of these thrombi as the initial lesion of atherosclerosis is accepted, there is an obvious connection between the presumptive failure of fibrinolysin to prevent these thrombi and the view that atherosclerosis is in some way the result of a failure of the body's defence mechanisms against the injury caused by the stress and strain of civilised life.

### SUMMARY

1. A fibrinolysin was demonstrated in over 90 per cent. of samples of fluid and incoagulable cadaver blood, all of which were free from fibrinogen.

2. The appearance of the lysin was considered to be part of the body's general reaction to injury. This accounted for its presence in the blood after death from a wide variety of causes, and for the frequency with which fluid and incoagulable blood was found at autopsy.

3. The fibrinolysin failed to appear after death from infections and cachexia, and this is the reason why in such cases the blood was found to be coagulated at autopsy.

4. The fibrinolysin is probably produced by the endothelial lining of the vascular channels and body cavities.

5. Cadaver fibrinolysin is a globulin with an optimum *pH* on the alkaline side of neutrality. Although it lyses fibrin it does not appear to affect fibrinogen.

6. Cadaver fibrinolysin differs from plasmin and the pancreatic proteinases, but may be related to the skin proteinase of Beloff and Peters and to the spontaneously active fibrinolysin of Macfarlane.

7. The possible physiological and pathological significance of fibrinolysin formation is outlined.

The greater part of this work was carried out in the Departments of Morbid Anatomy and Clinical Pathology, the Radcliffe Infirmary, Oxford, and the

# EPITHELIAL LESIONS IN THE RESPIRATORY TRACT IN HUMAN INFLUENZAL PNEUMONIA

M. STRAUB and J. MULDER

With the cooperation of A. ARENDS, J. A. R. VAN BRUGGEN  
and the late L. BIJLMER

*From the Rotterdam Municipal Institute of Pathology, the University Medical Clinic, Leyden, and the Institute of Pathology of the University Hospital of Groningen, the Netherlands*

(PLATES LXXII-LXXIV)

DURING the influenza pandemic of 1918 Askanazy (1919) described the "metaplasierende katarrh" of the tracheal and the bronchial epithelium, and expressed the opinion that it was more or less typical of this disease. By this he implied that the ciliated epithelium of trachea and bronchi had changed into a stratified undifferentiated epithelium, and he thought that this was probably entirely built up anew. In the same pandemic, Winternitz *et al.* (1920) described necrosis of the respiratory epithelium with consequent regeneration to an irregular stratified form throughout the entire respiratory tract, including the small and terminal bronchioles.

Studies on the pathology of experimental influenza in mice (Straub, 1937, 1939, 1940; Nelson and Oliphant, 1939) and ferrets (Francis and Stuart-Harris, 1938) have shown that in these animals the virus of inter pandemic influenza A causes necrosis of the goblet and ciliated cells of the epithelium of the respiratory tract, but spares the layer of basal cells. From this layer a regenerative process starts, causing the epithelium to become temporarily stratified. The results of these experiments give support to the idea that the influenza virus multiplies in the respiratory tract epithelium.

While we are well acquainted with the pathogenesis of this epithelial necrosis in mice and ferrets, we have a quite inadequate knowledge of the experimental lethal infections of those animals in which the typical hæmorrhagic and cedematous lung lesion is observed. Nor do we know whether this lesion is a real virus pneumonia or whether it must be explained in some other way. Continued research is urgently necessary here if we are to obtain a better understanding of the problem of influenzal pneumonia in man.

The epithelial lesions described above do not seem to have been found in pathological studies, after 1933, of cases of human influenzal pneumonia where the influenza virus was demonstrated in the lungs (Scadding, 1937\*; Stuart-Harris *et al.*, 1938; Stokes and Wolman, 1940; Himmelweit, 1943; Parker *et al.*, 1946).

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Our own observations during the 1939-46 period confirmed Askanazy's, and partly also those of Winternitz *et al.* A modified autopsy technique is, however, necessary; otherwise there is every likelihood that the epithelium of the trachea, bronchi and bronchioles will be lost or distorted in opening and washing out the air passages and in pressing on the lung parenchyma. The technique suggested is as follows. Autopsy should be performed as soon after death as possible. The trachea should not be opened, but should be carefully severed (without pressure) from the hilum of the lung and fixed *in toto*. As regards the lungs, portions of the inflamed lobes should be carefully excised with a very sharp knife and fixed intact. After a few days fixation, small pieces of trachea, bronchus and lung are excised for microscopic examination. Experience has shown that only by following this technique can the epithelium of the respiratory tract be prevented from developing artefacts which may make correct appreciation of the morbid anatomical changes impossible.

No investigations into the presence of influenza virus being possible in Rotterdam from 1939 until 1947, the pathological examination of the respiratory tract in different kinds of pneumonia took the form described above. In 12 cases it concerned a syndrome which, on account of the clinical symptoms and the macroscopic aspect of the lungs, could with reasonable probability be looked upon as secondarily infected influenzal pneumonia. In 7 of these cases two main types of epithelial lesion were found in the trachea and bronchi. One type of lesion showed epithelium which had been reduced by desquamation to one layer of flattened cells; in some of these, mitoses could be found (fig. 1). The other type showed regeneration starting from the basal layer of cells, with formation of an irregular stratified epithelium devoid of keratinisation and consisting of 2-6 rows of cells (figs. 2 and 3). Where there was no acute inflammation, the mucosa showed, under the basement membrane, both severe hyperæmia and infiltration with mononuclear cells. It is important that in none of the Rotterdam cases was the epithelium of the bronchioles observed to be affected, in spite of bacterial inflammation of their mucosæ (fig. 4).

In 6 of the 12 cases, both trachea and bronchi showed pseudo-membranous inflammation. In 8 cases the lung tissue was examined bacteriologically and in each the predominating organisms were staphylococci.

It goes without saying that these observations must be tested by combined virological and morphological examinations. It is true this was done on a somewhat larger scale by Parker *et al.*, but these authors did not pay sufficient attention to the existence of epithelial lesions in the trachea and larger bronchi; hence their pathological examination was incomplete.

During the big epidemic of influenza A in the Netherlands in 1941 (van Bruggen *et al.*, 1947), a fatal case of influenzal pneumonia

## TRACHEAL LESIONS IN INFLUENZAL PNEUMONIA



FIG. 1.—Tracheal mucosa of a clinically suspected case of influenzal pneumonia of four days' duration (22.2.46). The basal-cell layer is left, showing flattened cells. Below the basement membrane, hyperæmia with infiltration by lymphocytes and plasma cells.  $\times 200$ .



FIG. 2.—Tracheal mucosa of a clinically suspected case of influenzal pneumonia; duration of illness 8 days (27.2.46). Epithelium regenerating. Small ulcer in centre, with inflammatory infiltration in floor.  $\times 200$ .

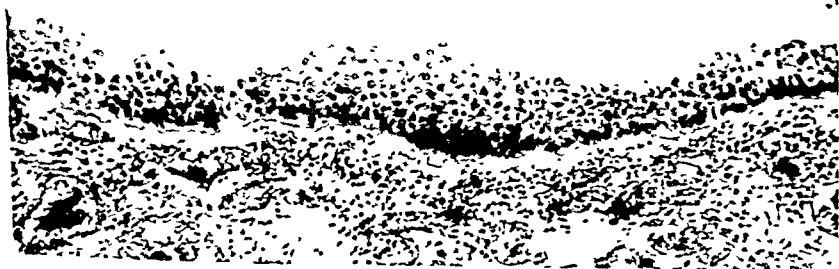


FIG. 3.—Tracheal mucosa of a clinically suspected case of influenzal pneumonia; duration 14 days (20.3.41). Regenerating epithelium of stratified (undifferentiated) type.  $\times 120$ .



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was investigated in the Medical Clinic of the University Hospital at Groningen.

### Case report

The patient, a woman (Kr.) aged 34, died on the eighth day of her illness (8th Feb. 1941) of broncho-pneumonia affecting all lobes of both lungs, with empyema in the right pleural cavity. During life, hæmolytic *Staphylococcus aureus* and hæmolytic streptococci were repeatedly cultivated from the sputum and from pus from the empyema. *Post mortem* the same micro-organisms were cultivated from the pus of the bronchogenic lung abscesses. *Hæmophilus influenzae* was lacking. The blood culture was sterile on the fourth day of illness. On that day an influenza virus, strain A in ferrets, was obtained from the sputum (table, expts. 2 and 3). The first and

TABLE

*Mouse protection tests with patient's serum and ferret sera against influenza A, strain WS (filtrate 1 in 10) \**

Expt.	Serum	Serum dilution			Control
		1 in 2	1 in 10	1 in 50	
1	Patient (Kr.) 4.2.41	++ +++	+++ D ++++	...	+++ D ++++
	Patient (Kr.) 8.2.41	...	— — +	— — —	D +++ D ++++
2	Ferret (inoculated with sputum; 3rd passage)	— — —	— — ++	— ++ ++	† D +++ D ++++
3	Ferret (inoculated with sputum; 3rd passage)	— — —	... — +	... ... ...	D +++ D +++ D +++
	Ferret (inoculated with ground lung)	+++ D ++++ D ++++	... +++ D ++++	... ... ...	D +++ D +++ D +++

\* Surviving mice killed after 6 days.

† Non-specific death.

+ to ++++ = different degrees of lung lesions.

D = died of influenzal pneumonia.

two subsequent passages showed fever on the third day, with rhinitis. The patient's serum showed an increase of immune bodies against the virus strain WS (influenza A) between the fourth and eighth days of illness (table; expt. 1). *Post mortem*, no virus could be demonstrated in the right lower lobe (table, expt. 3).

Recently the ferret sera of this case, which had been lost during the war, were found. They were freed from non-specific inhibitor by cholera filtrate and tested by the hæmagglutination-inhibition test. The "sputum ferret" serum showed a titre against PR8 of 192 and against Lee of  $<12$ . The "lung ferret" serum showed no antibody against PR8 or Lee (titre  $<12$ ). These experiments again prove that the case here described was one of influenza A.

The post-mortem diagnosis was: no tonsillitis; bluish-red hyperæmia of the mucous membrane of pharynx and palato-pharyngeal arches; slight pseudo-membranous tracheitis; extensive pseudo-membranous bronchitis; purulent bronchiolitis in all lobes of both lungs; confluent hæmorrhagic fibrino-purulent broncho-pneumonia with abscesses in all lobes; fibrino-purulent pleurisy; right-sided empyema.

### *Histopathology of the respiratory tract epithelium*

The epithelium of the trachea shows stratification without keratinisation of the cells, the number of the layers being from 4 to 6. Mitoses are not found (fig. 5). In the gland ducts the epithelium consists of 2 or 3 layers of flattened cells. There is slight infiltration of the epithelium with leucocytes and lymphocytes, as under the basement membrane. The mucosa shows severe hyperæmia. The epithelium of the greater bronchi has almost completely disappeared owing to the extensive pseudo-membranous inflammation of their walls. Sometimes, however, it is present, and it then consists of 1-4 layers of flattened or cubical cells (fig. 6). Mitoses are rare. Interruption of the epithelium by pseudo-membranous exudate is frequent. Generally there is severe infiltration of the epithelium by leucocytes.

A small bronchus (with cartilage) in the lingula of the left upper lobe was studied in serial section because of the absence of any severe cellular or fibrinous inflammation in its wall. The lumen is occupied by sero-fibrinous exudate with a relatively small number of leucocytes and many chains of streptococci. In places the epithelium has become desquamated, showing only 1 or 2 layers of flattened cells, some of them with pyknotic nuclei (fig. 7). In or below this layer, patches of cells with round and oval nuclei are seen which suggest that epithelial regeneration is in process (fig. 7). This is confirmed by the finding of mitoses (fig. 8). In one patch in the wall this regeneration has advanced to stratified (undifferentiated) epithelium (fig. 9), and there is severe infiltration with leucocytes. In some small bronchioles of the left upper lobe (lingula) the same regeneration of epithelium is seen. A small bronchus in the left lower lobe shows also a single layer of flattened cells together with severe inflammatory cell infiltration (fig. 10). A few mitoses in the basal-cell layer are found here. The epithelium of the small bronchioles in the left lower lobe, however, is mostly intact, in spite of the severe purulent inflammation of their

TRACHEO-BRONCHIAL LESIONS IN INFLUENZAL PNEUMONIA

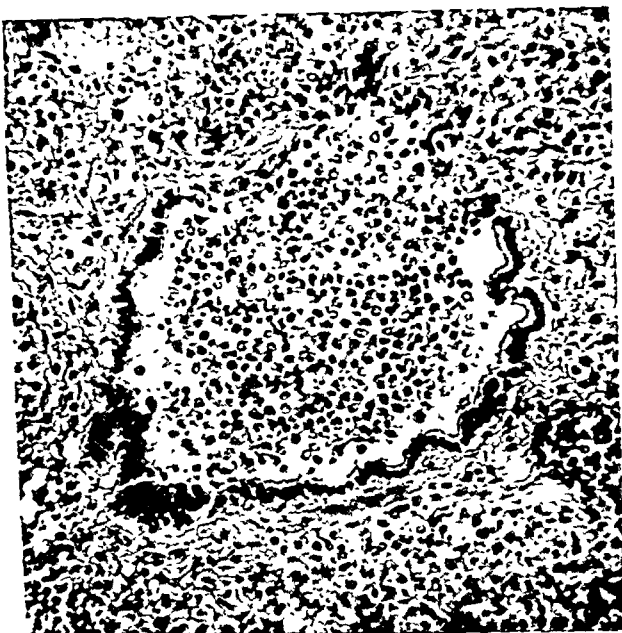


FIG. 4.—The same case as that of fig. 1. Purulent bronchiolitis. The epithelium is unchanged except for an area of ulceration (upper margin of bronchiole).  $\times 200$ .

FIG. 5.—Tracheal epithelium of a case of influenzal pneumonia (case Kr.). Duration of illness 8 days (8.2.41). Regenerated epithelium with stratification.  $\times 300$ .

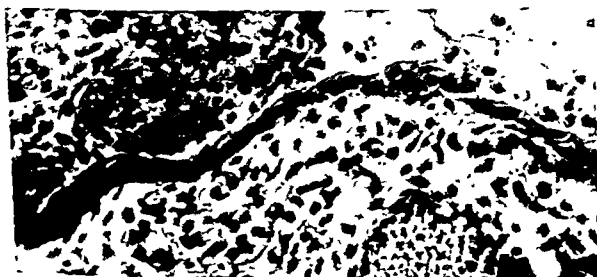
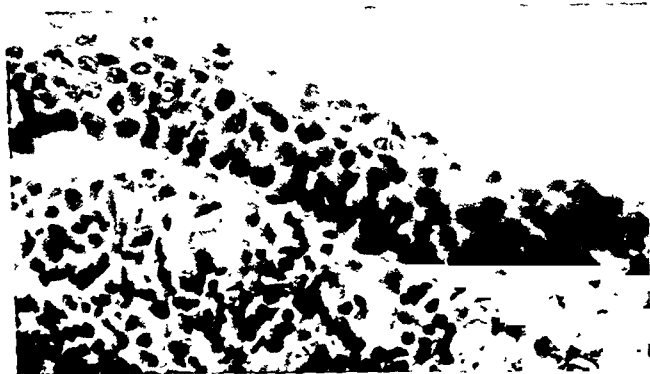


FIG. 6.—Case Kr. Remaining basal-cell layer of the epithelium of a large bronchus in the right lower lobe in the midst of an area of acute bacterial inflammation.  $\times 300$ .



## BRONCHIAL LESIONS IN INFLUENZAL PNEUMONIA



FIG. 7.—Case Kr. Small bronchus (with cartilage) in the lingula of the left lung, showing surviving layer of basal cells. In the middle, "young" epithelial cells. No acute bacterial inflammation in mucosa.  $\times 300$ .

FIG. 8.—Case Kr. The same small bronchus as in fig. 7. Mitosis in basal-cell layer.  $\times 300$ .



FIG. 9.—Case Kr. The same small bronchus as in figs. 7 and 8. Regenerating irregular epithelium. Two mitoses are seen. Severe infiltration with leucocytes.  $\times 300$ .

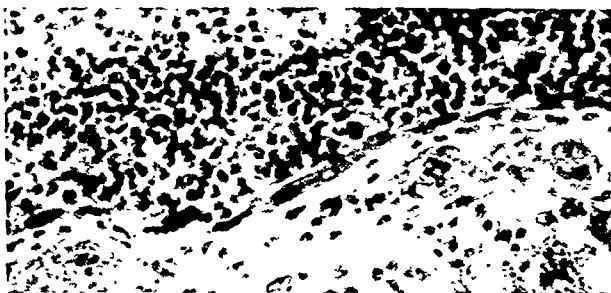


FIG. 10.—Case Kr. Small bronchus without cartilage. Purulent inflammation; remaining row of flattened basal cells.  $\times 300$ .

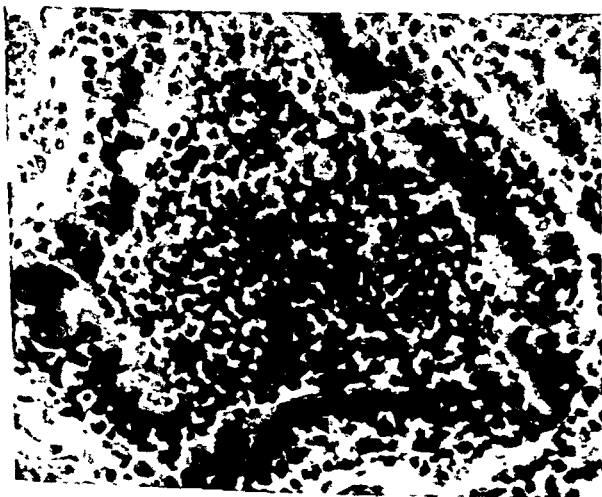


FIG. 11.—Case Kr. Bronchiole with pus-filled lumen. The epithelium itself is infiltrated with leucocytes but is otherwise undamaged.  $\times 300$ .





walls. The only exception to this is where purulent inflammation has broken through the wall (fig. 11). Most of the small bronchi and bronchioles are dilated.

### Discussion

An interpretation of the epithelial lesions described above cannot be given with absolute certainty. They look very much like the experimental influenzal epithelial lesions in mice and ferrets, and they partly correspond to the pandemic influenzal lesions in man described by Askanazy and by Winternitz. They might therefore very well be regarded as specific influenzal virus lesions. An alternative explanation would be that they are due to bacterial toxic action on the epithelial cells. Against this view it may be pointed out that the epithelium of most of the small bronchioles shows no similar lesion in spite of severe purulent (bacterial) inflammation, while the epithelial changes depicted in figs. 7-9 cannot properly be explained on the basis of a bacterial toxic lesion. In the first place it is improbable that such a lesion would spare the basal-cell layer: one never encounters this phenomenon in other situations where a purulent or pseudo-membranous inflammation destroys the bronchial wall. It is also important to notice that in spite of the severe bacterial inflammation figs. 8 and 9 show regenerating epithelial cells in addition to the leucocytic infiltration. It is difficult to explain pictures like these on the basis of a bacterial toxic lesion alone. Other purulent inflammations of the bronchi with severe cellular infiltration do not show comparable lesions, for instance those in *Hæmophilus* infections (personal observations).

In our opinion it is worth noting that Winternitz *et al.*, in their cases of pandemic influenza, describe extensive necrosis and pathological regeneration of the epithelium of the small and terminal bronchioles. It is possible that this is due to a difference in pathogenicity of the virus of pandemic and that of inter pandemic influenza.

We further feel that at post-mortem the virologist should not try to isolate influenza virus from the lung alone, but also, and particularly, from the mucous membrane of the trachea and bronchi.

### Summary

The observations of Askanazy and of Winternitz *et al.* on epithelial lesions of the trachea and bronchi in cases of influenza occurring during the 1918 pandemic were confirmed to a certain extent in various cases of clinically suspicious influenzal pneumonia seen during the 1939-47 period, as well as in a case of virologically confirmed inter pandemic influenza in 1941. A special autopsy technique for the trachea and lungs in influenza, necessary for the demonstration of these lesions, is described.

The epithelial lesions, which are more or less identical with those

described in mice and ferrets, consist in the disappearance of the goblet and ciliated columnar cells, the basal-cell layer being preserved and subsequently regenerating to a stratified (undifferentiated) epithelium. In all but one case the epithelium of the small bronchioles was found intact, even in a case where there was purulent bacterial bronchiolitis. This may constitute a difference from pandemic influenza, in which Winternitz *et al.* found necrosis of the bronchiolar epithelium also. The epithelial lesions now described are considered by the authors as being caused by the action of influenza virus. An alternative explanation of the epithelial lesions on the basis of bacterial toxic action cannot be completely refuted. Further combined virological, bacteriological and pathological investigations are necessary to determine the significance of these changes.

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## THE EFFECTS OF KAOLIN ON THE LUNGS OF RATS

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and

G. NAGELSCHMIDT

(PLATES LXXV-LXXVIII)

THE mineral kaolinite, of chemical composition  $\text{Al}_2\text{O}_3 \cdot 2\text{SiO}_2 \cdot 2\text{H}_2\text{O}$ , is one of the chief clay minerals. It is a layer lattice silicate built up of units of  $\text{Al}_2\text{Si}_2\text{O}_5(\text{OH})_4$  in a triclinic atomic arrangement which has recently been re-determined by Brindley and Robinson (1946). Kaolinite occurs in nature usually through alteration of feldspars by hydrothermal processes or by surface weathering. The mineral kaolinite forms the chief constituent of kaolins or china clays. These are produced industrially on a large scale and serve as raw materials in many industries. World production in 1937 was of the order of 3.2 million tons, of which a quarter came from the kaolin deposits in Cornwall and Devon. Other major producing countries were the United States, Czechoslovakia, Japan and Germany. The paper industry is the largest consumer of kaolin, followed by the ceramics, refractories, rubber, cement, paint and many other industries.

The older literature contains several references to pneumoconiosis produced by kaolin, *e.g.* Lemaistre (1894) and Hlava (1897). Most of the recent references to any industrial hazard arising from the inhalation of kaolin have been concerned with the pottery, ceramics and refractories industries, where kaolin is used in admixture with other mineral powders, notably feldspar and flint (Koelsch, 1930; Pancoast and Pendergrass, 1931; Legge and Rosencrantz, 1932; Gudjonsson and Jacobson, 1934; Möller, 1934; Quaintance and Morris, 1934; Hartmann, 1935; Langelez, 1935; Behneman, 1936; Kaestle, 1936). The possibility of pneumoconiosis occurring amongst workers in china clay, which is nearly pure kaolin, is discussed by Middleton (1936) who states (p. 61) that in the principal home of the industry in Cornwall "there is a general belief that no injury to health follows exposure to the dust".

Kaolin is also a prominent constituent of the rock strata associated with the South Wales coal measures. It occurs in considerable

concentrations in the dust of coal mines. King and Nagelschmidt (1945) concluded from a study of the analyses of coal miners' lungs that this kaolin was probably not a factor in the production of coal miners' pneumoconiosis, and Belt and King (1945) found a minimum of pathological change in the lungs of animals injected with those powdered strata from the coal mines which contain the largest amounts of kaolin. However, they also observed that a sample of commercial acid-washed kaolin produced some pathological change—more than that resulting from the natural kaolin-containing rock from South Wales. The possibility that acid treatment increases the pathogenicity of kaolin was explored by King, Gilchrist and Rae (1947).

The experiments of Kettle (1934) have an important bearing on the question of whether kaolin may be a factor in the production of pneumoconiosis. Kettle observed only a cellular reaction, with the formation of plaques of dust and cells in the alveoli, when kaolin was introduced into the lungs of guinea-pigs, but when mixed with dead tubercle bacilli kaolin caused the formation of fibrotic tissue in lesions very similar in structure to silicotic nodules. Siegmund (1935) also suggested the possibility that a tuberculous process may exaggerate the changes produced by kaolin and other silicious substances in the lung tissues.

The investigation here described was concerned with the effects produced by two forms of kaolin occurring in this country, both of which have at various times been thought to carry a possible industrial hazard, although no positive evidence has, as far as we know, ever been adduced. One was a fraction below  $1\ \mu$  equivalent diameter of a kaolin from Cornwall, the other was prepared from mineral matter of a bituminous coal from South Wales. This consisted originally of kaolin and calcium carbonate and the latter was dissolved by treatment with  $\text{CO}_2$ .

## MATERIALS AND METHODS

### *Mineral dusts*

1. *The South Wales kaolin* was obtained from a sample of the extraneous mineral matter from a bituminous mine (B. 29 (2) sinks 1.60; Hicks and Nagelschmidt, 1943). Besides the kaolinite, it contained carbonates in considerable amount.\* These were removed by passing water containing  $\text{CO}_2$  through a bed of 10 g. of the finely ground sample contained in a sintered glass crucible. The process was continued until the loss of weight of the residue was less than 0.01 g. It took approximately 100 hrs. before such conditions were obtained. The loss in weight when constancy was obtained was 3.32 g., equivalent to 33.2 per cent. of the original sample. The  $\text{CO}_2$  in the residue so obtained was "adsorbed" 1.02 per cent., "fixed" 0.74 per cent. It is therefore unlikely that the residue contained more than 2 per cent. of carbonates.

\* The original sample contained 16.12 per cent. of  $\text{CO}_2$  and the carbonates calculated from the analysis of the ash were:

$\text{CaCO}_3$	.	.	.	16.84	per cent.
$\text{MgCO}_3$	.	.	.	10.82	"
$\text{FeCO}_3$	.	.	.	8.02	"
				<u>35.68</u>	"

REACTION OF LUNGS TO KAOLIN

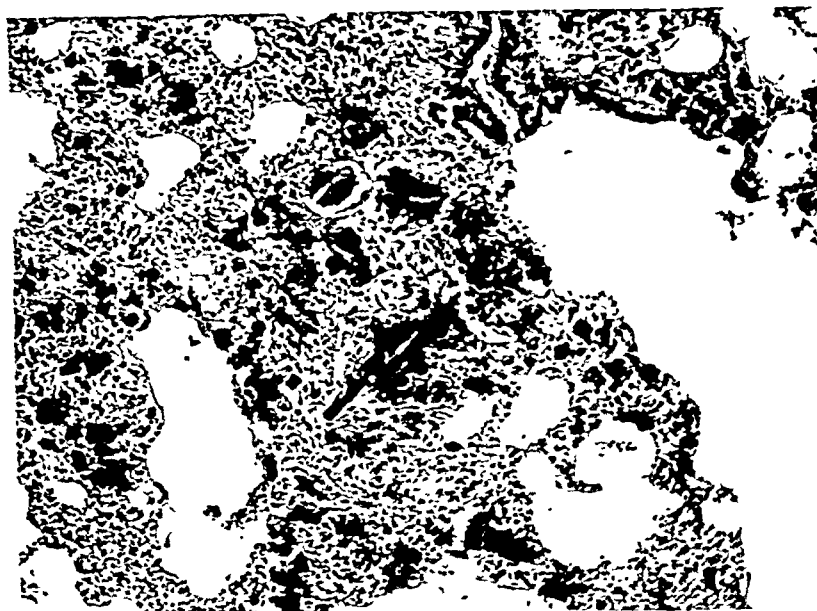


FIG. 1.—South Wales kaolin. Rat lung after 90 days' survival, showing collections of kaolin in coarse aggregates with a little reaction. H. and E.  $\times 135$ .

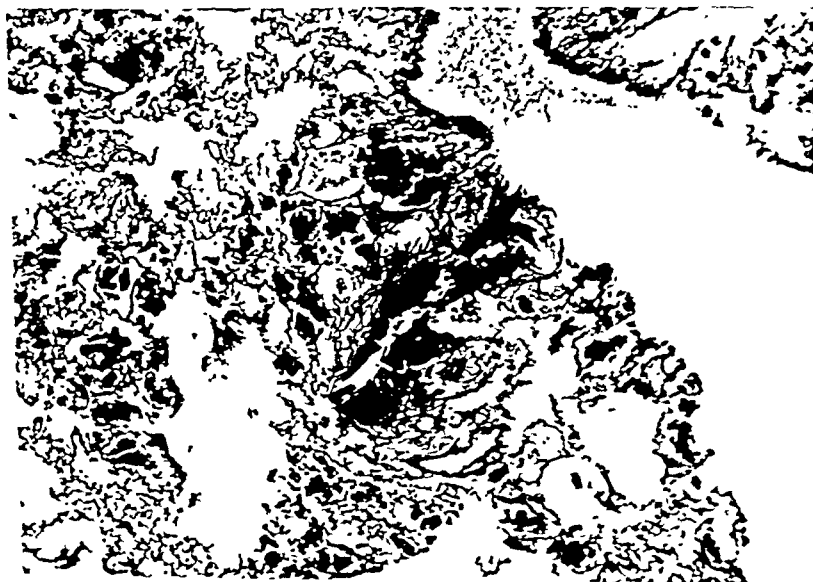


FIG. 2.—Similar section showing minimal reticulin production. Silver impregnation.  $\times 135$ .



REACTION OF LUNGS TO KAOLIN

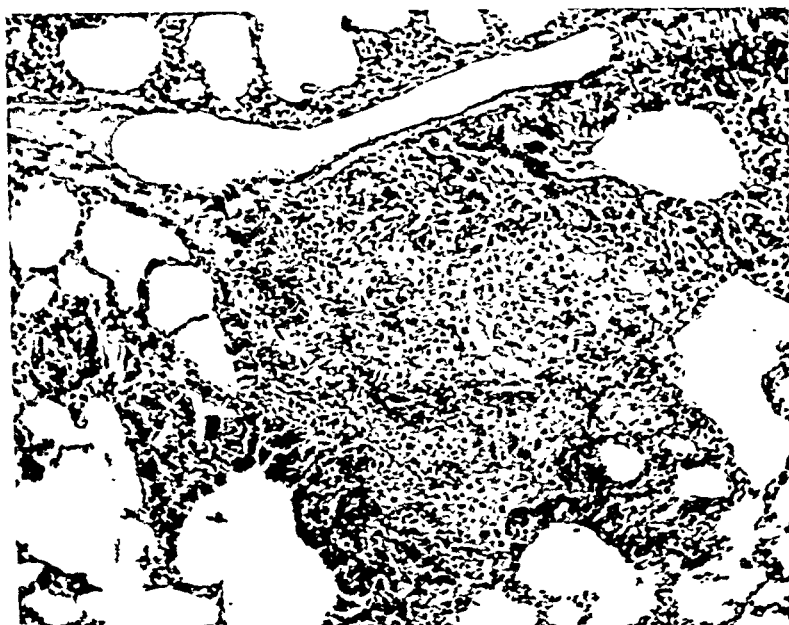


FIG. 3.—Untreated Cornish kaolin. Rat lung after 161 days' survival, showing collections of macrophages filled with kaolin. H. and E.  $\times 105$ .



FIG. 4.—Similar section showing absence of any reticulin production. Silver impregnation.  $\times 105$ .

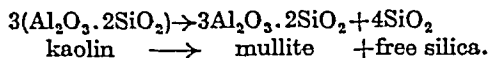




2. *The Cornish kaolin* consisted of two samples.

(a) *Untreated kaolin* was prepared as follows. Fifty g. of a commercial fine kaolin were suspended in water and the fraction remaining in suspension at 5 cm. height after 24 hours was removed twice. The clay was flocculated with the minimum amount of hydrochloric acid required to bring the suspension to a pH between 3 and 4, collected on a Buchner filter, washed with water and dried.

(b) *Ignited kaolin* is used as a raw material for refractories. Two-thirds or more of the silica is present in the ignited product as silica glass finely distributed in mullite. The decomposition of the kaolin on heating is illustrated by the following equation



The free silica forms a glass with minor amounts of alkalis and other constituents present, and, under equilibrium conditions, this glass contains about 5 per cent. of  $\text{Al}_2\text{O}_3$ . The action of this material on the lung is completely unknown. A sample was prepared for animal experiment as follows. Two hundred g. of a ground sample of calcined kaolin were shaken up with water and the material remaining in suspension at 5 cm. height after 6 hours was repeatedly syphoned off. After settling and decanting, the residue was dried, giving about 30 g. of material under 2  $\mu$ .

3. *Quartz* was used in a control experiment for comparison with the kaolins. The sample used was from a commercial source (Messrs Colin Stewart, Ltd., to whom we are grateful for a supply). On analysis it contained 97.1 per cent.  $\text{SiO}_2$ . The size distribution of the particles is shown in table I:

TABLE I  
*Size distribution of quartz particles used in a control experiment*

Size ( $\mu$ )	Per cent. (by no.)
Under 0.25 . . .	7.7
„ 0.5 . . .	28.9
„ 1 . . .	67.1
„ 1.5 . . .	83.6
„ 2 . . .	91.9
„ 3 . . .	98.0
„ 4 . . .	99.0
„ 5 . . .	99.5
„ 6.8 . . .	100.0

#### *Silica solubilities of mineral dusts*

The amounts of silica released into solution from the above dusts were determined by the procedure described by King (1945, 1947). The results are given in table II. The silica solubilities of the kaolin are all very low, and similar to those recorded for the impure kaolins and shales used in previous investigations in this laboratory. The solubility of the commercial quartz (3.4 mg.  $\text{SiO}_2$ /100 ml.) is considerably greater than that of the kaolins, but is less than that of the purified samples of quartz described in previous papers. Its solubility was depressed to almost nothing by admixture with 1 per cent. of powdered aluminium.

*Experimental procedure*

The animal experiments were conducted along the lines previously described (Belt and King, 1915; King, Gilchrist and Rae, 1947). The technique was that of Kettle and Hilton (1932). Black and white rats of 200 g. weight were lightly anaesthetised with ether and the trachea exposed by blunt dissection. The powdered kaolins, suspended in saline containing 3 per cent. of milk, were

TABLE II

*Silica solubilities of kaolins and quartz*

(0.2 g. of mineral dust shaken with 10 ml. of buffered Ringer solution (pH 7.4) for 48 hrs. at 37° C.)

	Amount of dissolved silica (mg. SiO <sub>2</sub> /100 ml.)
South Wales kaolin . . . .	0.6
Cornish kaolin, untreated . . . .	0.4
" " ignited . . . .	0.6
Quartz, commercial (C.S.) . . . .	3.4
Quartz + aluminium powder . . . .	0.1

sterilised by steaming and injected into the lungs through a sharp needle inserted into the trachea. Each ml. of the mixture contained 50 mg. of kaolin. Between 1 and 1.2 ml. (i.e. 50-60 mg. of kaolin) were injected into each animal, ten rats being used for each sample. There were a few operation casualties, but most of the animals survived and lived for varying periods up to 6 months, when the remaining rats were killed.

## RESULTS

*South Wales kaolin.* Nine of the 10 rats were available for examination, the remaining rat having died and been eaten by its mates. By the 40th day the particles of kaolin were collected into aggregates, which in turn were collected into foci in the lungs. The aggregates lay partly within the alveoli and partly in the interstitial tissue. Up to the 60th day no fibrous reaction was produced. After 60 days there was a strictly local reticulinosiis in the parts of the lungs where the kaolin lay (figs. 1 and 2). This reticulinosiis was slight in amount and appeared to be mainly a walling off of the foreign material. There was never any fibrosis or emphysema. So far as this experiment is concerned, South Wales kaolin does not appear to produce any fibrosing lesion comparable with silicosis.

*Cornish kaolin (untreated).* Of the 10 rats, 6 died on the 1st day, one on the 2nd, one on the 9th, one on the 157th and one on the 161st. Those dying in the first few days showed the presence of the kaolin. The rat which died on the 157th day showed neither foreign particles nor reaction. The rat which survived for 161 days showed numerous small pneumonia-like foci of consolidation throughout both lungs (figs. 3 and 4). In these foci the alveoli were filled with large macrophages which contained some dust. There was no increase of reticulin. It would be unsafe to draw conclusions from this experiment, but the fact that two animals survived for more than 100 days without any fibrous reaction suggests that Cornish kaolin does not produce severe fibrosis.

REACTION OF LUNGS TO KAOLIN

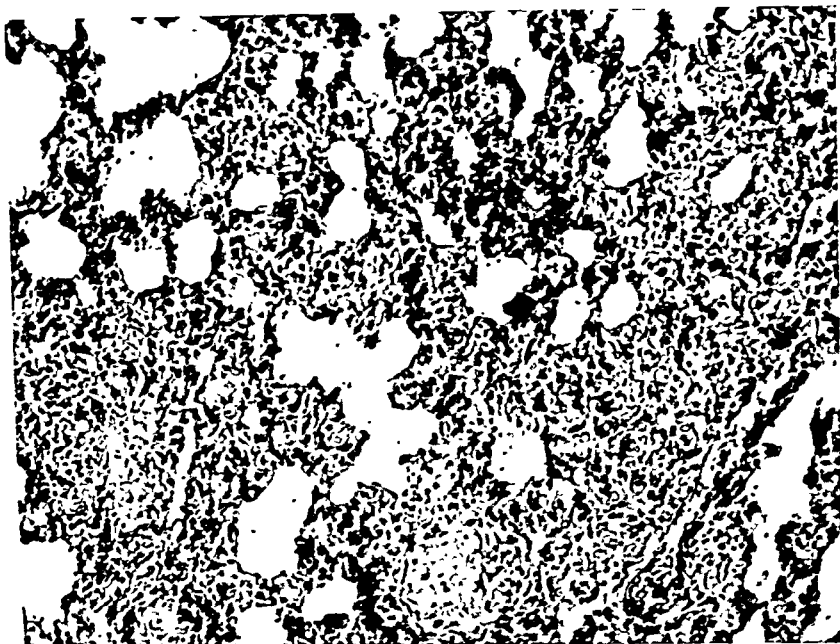


FIG. 5.—Ignited Cornish kaolin. Rat lung after 139 days' survival, showing a cellular reaction. H. and E.  $\times 145$ .

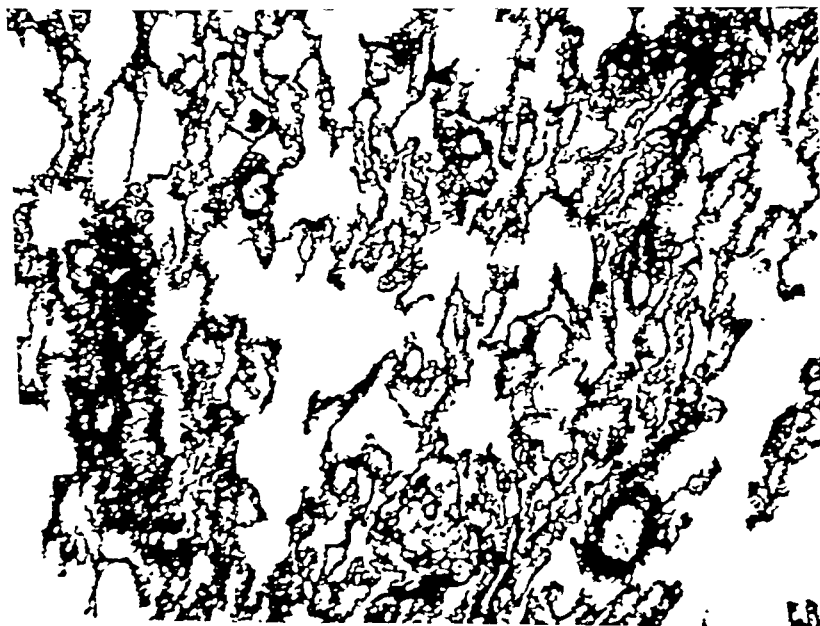


FIG. 6.—Similar section showing slight perivascular reticulin production. Silver impregnation.  $\times 145$ .



REACTION OF LUNGS TO C.S. QUARTZ

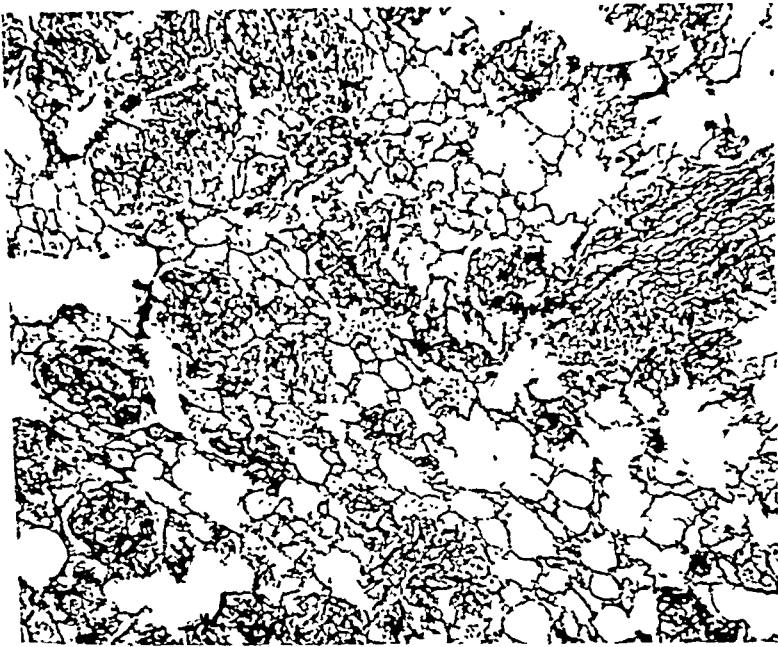


FIG. 7.—C.S. quartz. Rat lung after 68 days' survival, showing foci of reticulin production. Silver impregnation.  $\times 55$ .



FIG. 8.—C.S. quartz. Rat lung after 121 days' survival, showing coalescing nodules of silicosis. Silver impregnation.  $\times 55$ .



*Cornish kaolin* (ignited). Only 4 of the 6 rats used in this control experiment were available for examination: these had survived for 14, 28, 73 and 140 days respectively. In the first three there was a simple phagocytic reaction without reticulinosi. In the rat which survived for 140 days there was a phagocytic reaction in the perivascular connective tissue, associated with a strictly local reticulinosi (figs. 5 and 6). It is unsafe to draw conclusions from this single animal, but the result suggests that ignited kaolin can produce some local reticulinosi but not fibrous silicosis.

*Quartz* (commercial C.S.). Five of the 6 rats used in this experiment were available for study: they had survived for 68, 121, 130, 207 and 240 days respectively. All showed severe nodular silicosis. At 68 days there was a patchy but not nodular cellular consolidation, consisting of fibroblasts and macrophages. With silver impregnation considerable reticulinosi was visible in the form of fine fibrils forming a tangled web in the consolidated areas (fig. 7). These lay mainly around vessels and bronchi, but also occurred free in the parenchyma. At 121 days the lesions had become typical silicotic nodules (fig. 8). They were densely collagenous in the centre but with some cellular activity (of fibroblasts and macrophages) at their periphery. By 200 days these rounded nodular lesions had lost any cellularity and formed spherical masses of acellular collagen.

### SUMMARY

The pathogenicity of samples of South Wales and Cornish kaolin, in comparison with quartz, has been tested in rats by the intratracheal insufflation of suspensions of the powdered minerals into the lungs. The kaolins produced only a very mild reticulinosi reaction not comparable with the silicosis produced by quartz. A specimen of ignited kaolin, which contained amorphous silica, produced slightly more reticulinosi but no fibrous silicosis.

We are grateful to the Medical Research Council for a grant to defray part of the expenses of this investigation.

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616—018—021 . 6 : 547 . 963 . 32 (pentose nucleotide)

## TISSUE CHANGES IN MICE TREATED WITH PENTOSE NUCLEOTIDES AND RELATED COMPOUNDS

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(PLATE LXXIX)

THE results of preliminary investigations into the action of the pentose nucleotides on tissues of normal and tumour-bearing mice have already been described (Barker, Gulland and Parsons, 1946; Parsons, Gulland and Barker, 1947). Further experiments have confirmed these results, and examination of the biological changes induced in normal, irradiated and grafted mice by injection of uridylic acid, or two or more of the nucleotides in various combinations, has been carried out. Preliminary experiments have also been undertaken with adenosine, uracil and uridine. Results of these experiments are set out below.

### MATERIAL AND METHODS

#### *Groups of experimental mice*

1. Tumour-bearing stock and pure-line mice treated with pentose nucleotides and their corresponding controls (650).
2. Normal mice treated with pentose nucleotides (227).
3. X-radiated mice treated with pentose nucleotides (86).

Stock and pure-line (CBA and C 57) normal and tumour-bearing mice were used, the tumour-bearing pure-line mice being grafted with homologous methylcholanthrene sarcomas, the grafted stock mice with the heterologous Crocker sarcoma. Though the latter, when treated with guanylic acid, showed diminution in size and weight of the tumours as compared with the controls, the results were inconsistent and experiments with stock mice were discontinued.

#### *Tumours employed*

*Methylcholanthrene sarcoma in CBA mice.* This homologous tumour and its systemic effects have already been described (Parsons, 1942, 1943).

*Methylcholanthrene sarcoma in C 57 mice: systemic effects.* This sarcoma has been found to induce only a moderate leucocytosis in grafted mice, and neither amyloid nor myeloid conditions of the liver and spleen have been noted. Reticulosis and plasmocytosis of lymph glands is common, and iron-reacting

1946; Parsons, Gulland and Barker, 1947) and the results of experiments generally are given in table II. Though iron-reacting deposits occurred in spleens of untreated grafted C 57 mice, yet histological examination of spleens of tumour-bearing mice injected with adenylic, guanylic or cytidylic acid suggests that considerably larger amounts of iron-reacting material occurred in these than in those of the untreated controls. No quantitative estimation of the total iron content has yet been made. Injection of uridylic acid appeared to diminish the

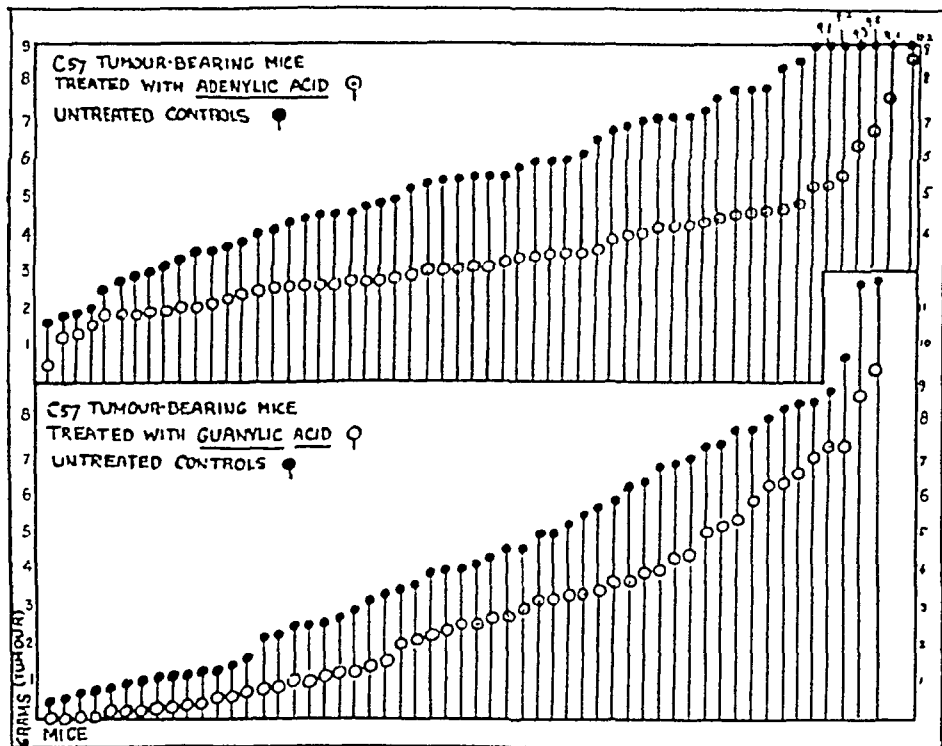


FIG. 1.—Chart showing tumour weights in C 57 sarcoma-bearing mice treated with adenylic or guanylic acid and the corresponding controls. Each upright indicates a treated and a control mouse.

amount of iron-reacting deposits, which were frequently entirely absent. In two mice treated with guanylic acid amyloid infiltration of the spleen was observed; this condition was not noted in any of the control mice.

The average splenic weight in sarcoma-bearing mice treated with adenylic or cytidylic acid was low or within the normal range and the number of giant cells per unit area was diminished. In contrast, spleens of mice treated with guanylic or uridylic acid showed an increase in weight and in the number of giant cells.

Injection of the nucleotides did not appear to have much effect on the leucocytosis which accompanies the growth of grafted C 57 sarcoma. Table III gives representative white-blood-cell counts from

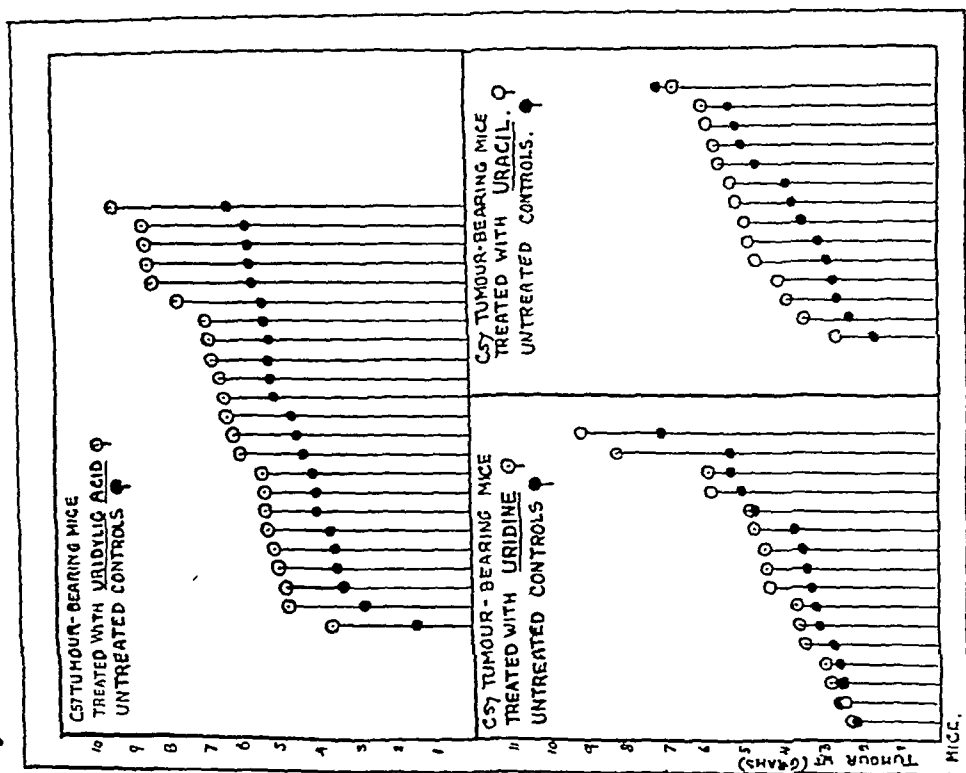


FIG. 3.—Chart showing tumour weights in C57 sarcoma-bearing mice treated with uridylic acid, uracil or uridine, and the corresponding controls.

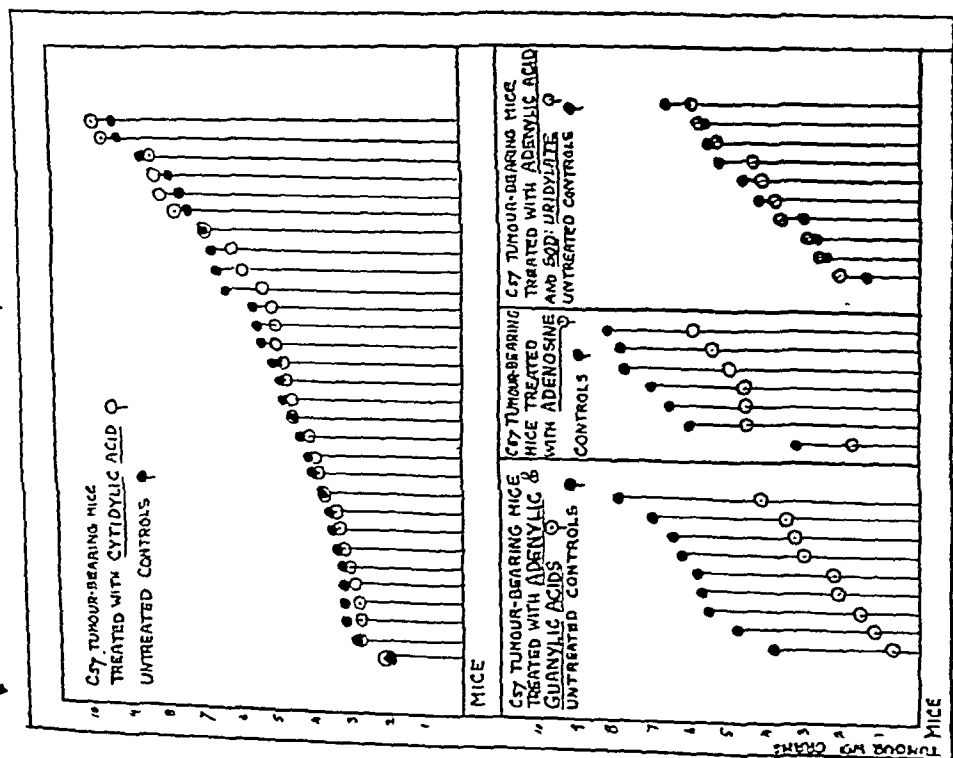


FIG. 2.—Chart showing tumour weights in C57 sarcoma-bearing mice treated with cytidylic acid, or with adenosine, adenylic acid, or adenylic acid & guanylic acids, and the corresponding controls.

simultaneously with cytidylic acid very high blood counts were obtained, the leucocytosis reaching leukæmic proportions in many cases. Total counts of 100,000-200,000 white blood cells per c.mm. have been recorded (fig. 9). In these cases absolute values of

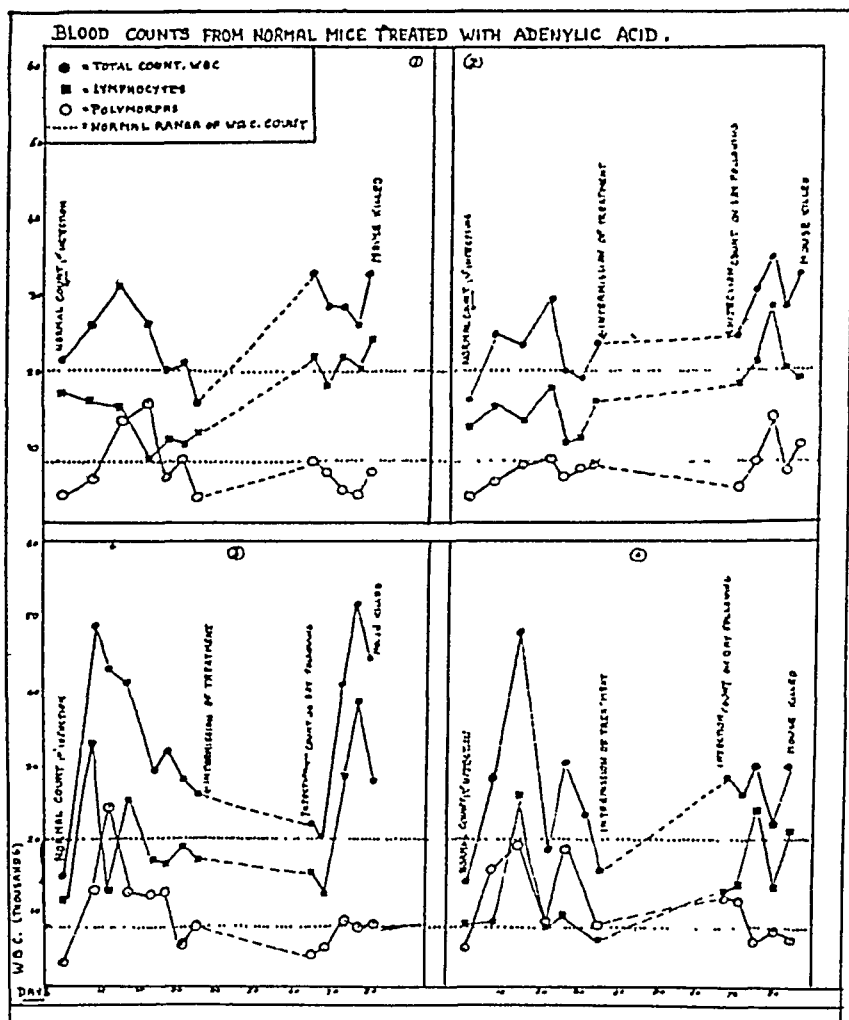


FIG. 4.—Chart showing representative blood counts in stock mice treated with adenylic acid.

lymphocytes were increased, the polymorph count varied and might occasionally cross the lymphocyte line, and an eosinophilia of moderate degree was present.

Where sodium uridylate and adenylic acid were injected simultaneously, their combined action on the blood varied. In some cases the total count remained within the normal range and the relationship of lymphocytes and polymorphs was unchanged: in others a leuco-

# BLOOD COUNTS FROM NORMAL MICE TREATED WITH GUANYLIC ACID.

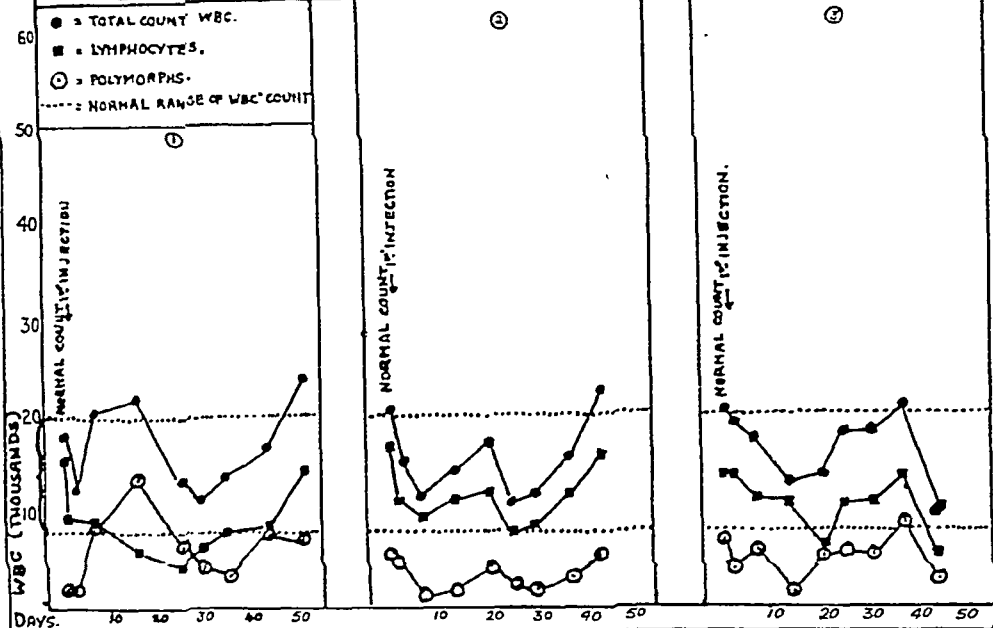


FIG. 6.—Chart showing representative blood counts from three young stock mice (same litter) treated with guanylic acid.

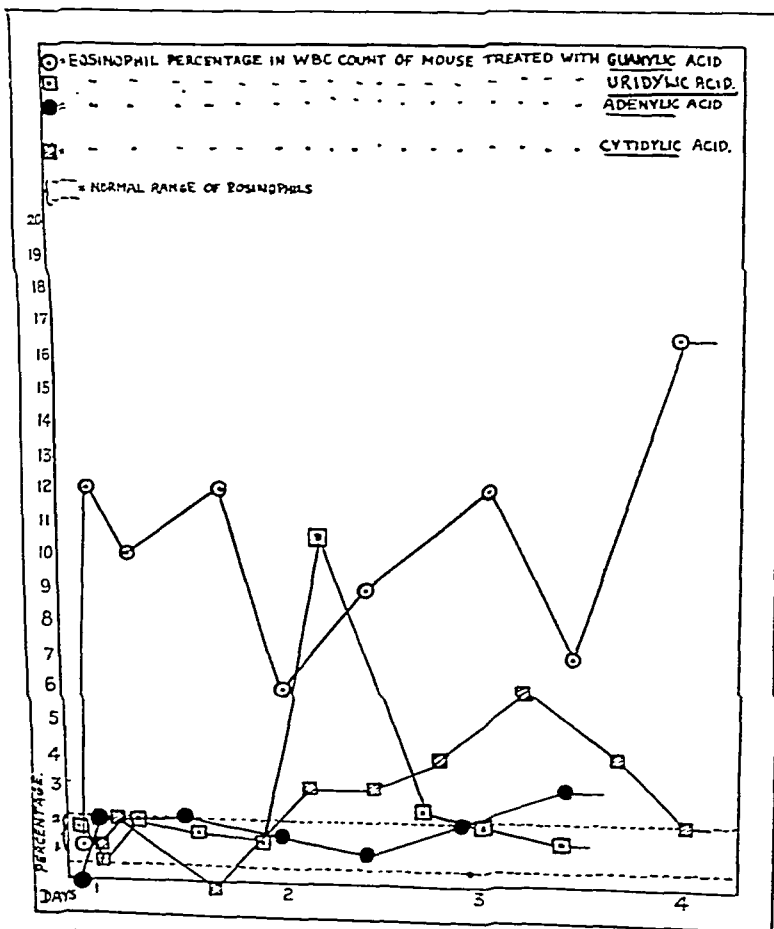


FIG. 7.—Chart showing representative eosinophil counts in mice treated with adenylic, guanylic, cytidylic and uridylic acid.

cytosis of late appearance in treatment and rising to 40,000-60,000 white blood cells per c.mm. occurred. In these cases the absolute values of both lymphocytes and polymorphs were increased. Where, however, guanylic acid was administered together with uridylic acid, a leucocytosis of considerable proportions occurred and might reach

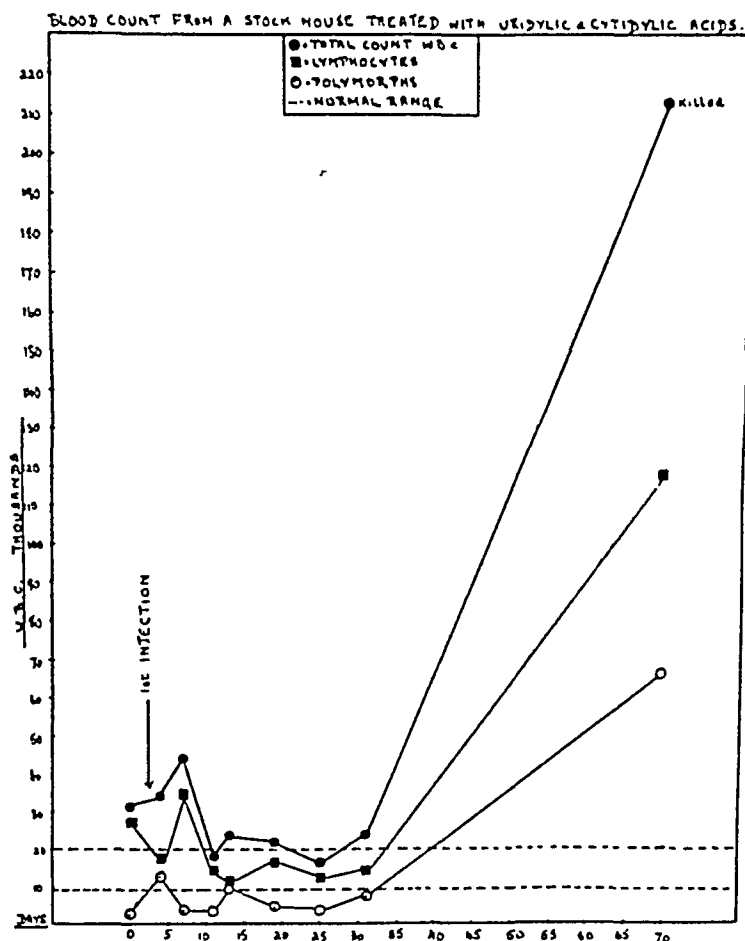


FIG. 9.—Chart of blood counts from mouse treated with uridylic and cytidylic acids injected simultaneously.

60,000-80,000 white blood cells per c.mm., the rise in the total count being due to a rise in the absolute values of lymphocytes only (fig. 10).

The eosinophilia mentioned above frequently appeared to bear a relationship to the changes in the polymorph count, a rise in the eosinophils coinciding with a fall in the polymorphs. This was noteworthy in the group of mice injected with guanylic and cytidylic acids simultaneously. Treatment with these compounds induced a leucocytosis within 24 hours of the first injection, which, with fluctuations, remained at a high level and might reach 50,000-60,000 white

blood cells per c.mm. It was observed that the increase or decrease of the polymorph count coincided with a decrease or increase of eosinophils. Since guanylic acid appeared to stimulate the production of eosinophils and cytidylic acid to increase the other granular cells these results suggest that at one phase during treatment the purine nucleotide exerted a dominant action, at another the pyrimidine nucleotide.

Mice which had adenylic and guanylic acids injected together showed blood changes characteristic of guanylic acid. The total

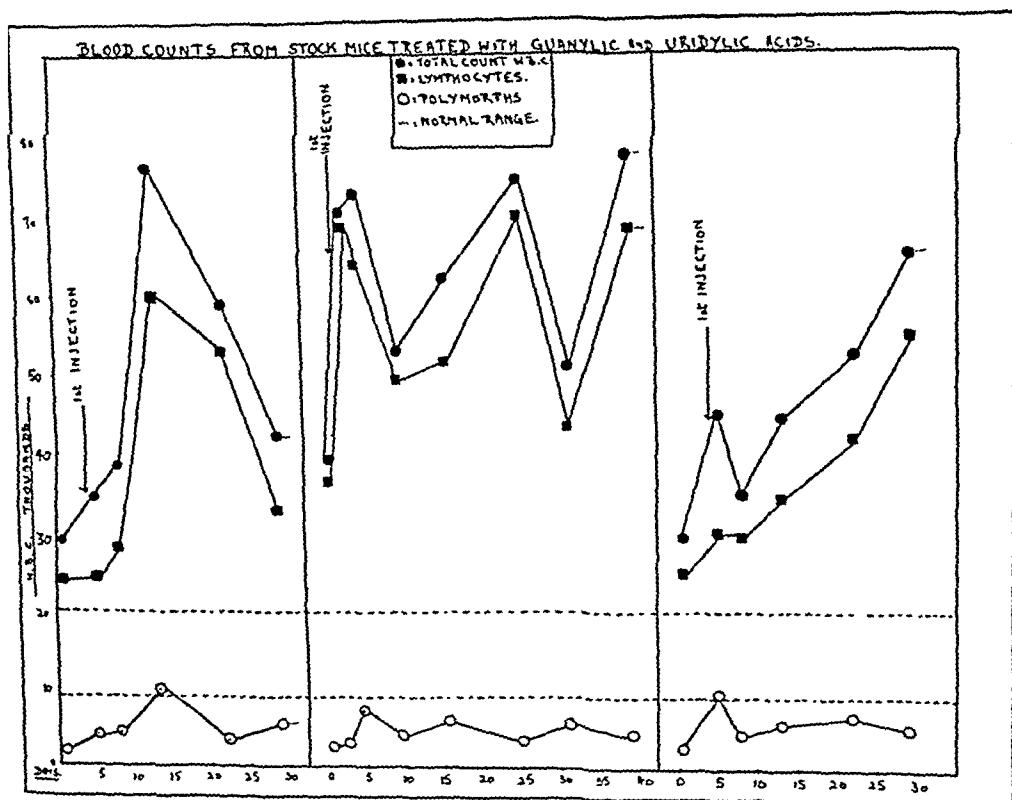


FIG. 10.—Chart showing representative blood counts from mice treated with guanylic and uridylic acids injected simultaneously.

counts tended to remain at a very low level, a feature which might be due to the combined action of the two inhibitory purine nucleotides on the blood-forming tissues. The relationship of polymorphs to lymphocytes was rarely altered. When, however, adenylic and cytidylic acids were administered simultaneously a moderate leucocytosis usually occurred, which might rise to 50,000 white blood cells per c.mm. Absolute values of lymphocytes were frequently diminished, but those of polymorphs and immature myeloid cells were increased. Eventually the polymorph line tended to cross the lymphocyte and to be maintained at a higher level for considerable periods (fig. 11).



These nucleotides injected together appeared to be the two compounds definitely stimulating and sustaining an increased output of polymorph leucocytes. Since both adenylic and cytidylic acids had been found to increase the number of immature myeloid cells in the blood this reaction was to be expected.

Blood changes in mice treated with uracil or uridine appeared similar to those noted in mice injected with uridylic acid. Adenosine appeared to exert an action similar to adenylic acid.

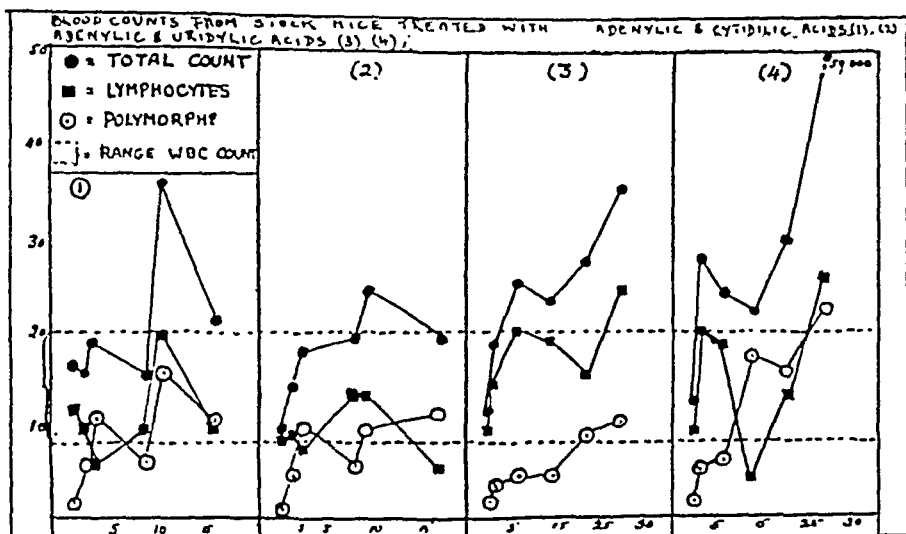


FIG. 11.—Chart showing representative blood counts from mice treated with (1 and 2) adenylic and cytidylic acids injected simultaneously, and (3 and 4) adenylic and uridylic acids administered together.

**Spleen and liver.** Table IV sets out the splenic changes occurring in normal mice treated with the four nucleotides. Injection of two compounds simultaneously seemed to cause more toxic effects than either administered separately. This was marked in mice treated with the two purine compounds, where the splenic weight was much diminished and focal necrosis of spleen, liver and lymph glands was common. The mortality among these mice was high. These effects might be due to the combined inhibitory action of purine nucleotides tending to arrest cellular activities, as in their action on the blood. Focal necrosis was, however, observed in the spleen and liver of mice of all groups, but its incidence was rare. A considerable increase of splenic giant cells was found in mice treated with guanylic or uridylic acid, or either of these in combination with adenylic or cytidylic acid.

Amyloid infiltration of the spleen and liver was found in isolated cases in most of the groups of mice as set out in table IV. Iron-reacting deposits were abundant in the spleen of mice treated with cytidylic acid but scanty or absent in the spleen of mice injected with uridylic acid, uracil or uridine. Pigment giving the reactions of

melanin occurred in the spleen of mice of all groups, but was most abundant in mice treated with guanylic acid, or guanylic acid injected with either of the pyrimidine nucleotides.

TABLE IV

*Splenic changes in normal mice injected with pentose nucleotides and related compounds, singly or in combination*

Compound	No. of mice	Average splenic wt. (g.)	Average no. of giant cells per unit area	No. of mice showing amyloid change	Iron-reacting deposits	Pigment (melanin)	Focal necrosis
Adenylic acid . . .	32	0.24	6.1	3	+++	±	Rare
Guanylic acid . . .	21	0.18	9.5	3	+++	±	"
Cytidylic acid . . .	10	0.16	7.0	0	++	±	±
Uridylic acid . . .	18	0.42	10.1	1	±	±	Occasional
Adenosine . . .	7	0.26	2.1	0	±	±	±
Uridine . . .	9	0.29	8.1	0	±	±	±
Adenylic and cytidylic acids	16	0.13	3.5	0	++	±	Rare
Adenylic and guanylic acids	16	0.12	9.5	2	+++	±	Frequent
Adenylic and uridylic acids	5	0.34	5.5	0	+	+	±
Guanylic and cytidylic acids	15	0.54	8.8	3	±	++	±
Guanylic and uridylic acids	15	0.34	3.5	1	+	++	±
Cytidylic and uridylic acids	29	0.54	9.1	2	±	±	Occasional

**Lymph glands.** The lymph nodes of mice treated with pentose nucleotides might be diminished, normal or increased in size. Considerable enlargement was noted in mice treated with guanylic acid injected with either of the two pyrimidine nucleotides. Histologically the changes induced in the glands of mice treated with the four nucleotides were similar to those described in the glands of irradiated mice, in mice undergoing treatment with carcinogenic compounds or in those bearing primary or grafted sarcomas (Parsons, 1938). In mice treated with simultaneous injections of adenylic and guanylic acids reticulosis was a marked feature, with diminution of lymphoid tissue. The reticulum cells were frequently of great size and stained a characteristic cherry-red colour with Pappenheim. They usually contained a small central nucleus, but multinucleated giant cells or giant cells with multilobulated nuclei were also occasionally found. These giant reticulum cells were highly phagocytic and contained red and white blood cells, plasma cells and, in many cases, iron-reacting deposits. Such cells occurring in the glands of mice treated with nucleotides resemble closely the large reticulum cells of similar staining reaction and phagocytic function noted in the glands of mice bearing primary methylcholanthrene sarcomas. In this the action

of the nucleotides on the tissues is similar to that of sarcomatous growth in the body.

Iron-reacting material was found in the glands of mice treated with the purine nucleotides and with cytidylic acid, to a much less degree in those injected with uridylic acid, uridine or uracil.

Preliminary experiments to test the combined action of adenylic, guanylic and cytidylic acids have suggested that these three compounds together exert little effect on the total blood count, which is generally maintained within the normal range. Absolute values of polymorphs are increased and eosinophilia is frequent. Splenic changes are being further investigated, as is also the combined action of these nucleotides on tumour growth.

### *X-irradiated mice treated with pentose nucleotides*

Tissue changes in irradiated mice injected with the nucleotides were similar to those noted in normal mice treated with these compounds. Table V sets out the splenic changes induced in the irradiated

TABLE V

*Splenic changes in irradiated mice injected with pentose nucleotides*

Compound Injected	No. of mice	Average splenic wt. (g.)	Average no. of giant cells per unit area	No of mice showing amyloid change	Iron-reacting deposits	Pigment (melanin)	Focal necrosis
Adenylic acid . .	13	0.22	7.2	0	+++	Rare	±
Guanylic acid . .	17	0.26	23.9	9	++	Rare	±
Cytidylic acid . .	10	0.30	4.7	1	±	±	±
Adenylic and guanylic acids	20	0.17	17.7	2	+	±	±
Adenylic and cytidylic acids	4	0.15	13.6	2	+	±	±
Guanylic and cytidylic acids	5	0.29	2.4	0	±	+	...
Uridylic and cytidylic acids	17	0.40	11.1	0	±	+	±

animals. It has been previously shown (Parsons and Warren) that X-radiation causes a diminution of splenic giant cells per unit area. It will be seen, however, that in 5 of the 7 groups of irradiated mice treated with the pentose nucleotides the number of giant cells was markedly increased (fig. 12). Irradiation also appears to increase the incidence of amyloid infiltration, this condition being marked in mice treated with guanylic acid, where the percentage of cases rose from 14.2 in non-irradiated mice to 52.9 in irradiated. Focal necrosis was common, and heavy iron-reacting deposits were found in the spleen and lymph glands of mice treated with the two purine nucleotides.

It was also noted that the leucopænia which is usually present in generally irradiated mice and rises slowly to normal over a period

CHANGES INDUCED BY PENTOSE NUCLEOTIDES



FIG. 5.—Axillary gland of mouse treated with adenylic acid, showing dark (prussian-blue-positive) areas at periphery with broad bands running down to hilum. Potassium ferrocyanide and hydrochloric acid.  $\times 45$ .

FIG. 8.—Spleen of mouse 1219 treated with guanylic acid, showing areas of amyloid infiltration encircling the malpighian bodies. H. and E.  $\times 50$ .

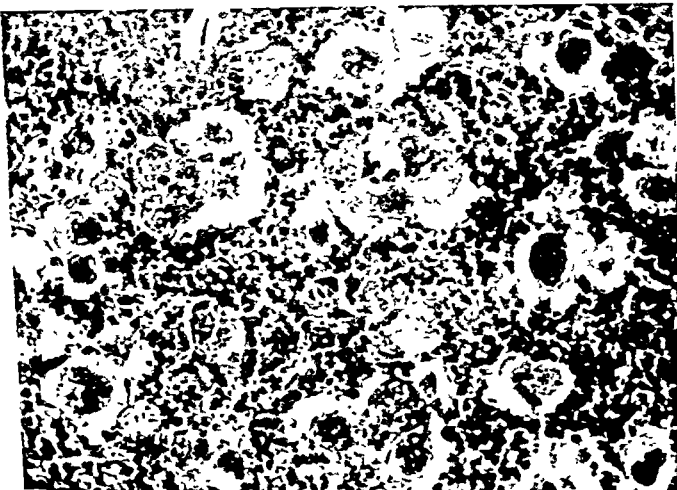


FIG. 12.—Spleen of irradiated stock mouse treated with sodium guanylate, showing marked increase in the number of giant cells per unit area. H. and E.  $\times 290$ .



of 10-16 days, was of shorter duration in mice treated with the nucleotides immediately after X-radiation. In these mice the leucocyte count rose steeply in 6-10 days and was typical of the particular nucleotide employed. It was accompanied by an increase in the polymorph count.

### DISCUSSION

From the above observations on the action of the pentose nucleotides on tissues of normal and tumour-bearing mice, the biological activities of these compounds would appear to fall into three main groups, namely: (a) those involving their inhibitory or growth-promoting action, more especially with reference to neoplastic changes; (b) those inducing systemic effects closely resembling those induced by tumour growth in the body; (c) those specific actions and reactions of the individual compounds, either associated or dissociated in the cell, whereby a balance is normally maintained in cellular activities and responses to stimuli regulated.

Recent work has increasingly shown the relationship of nucleic acid to tumour growth, and the observations of Caspersson and Santesson (1942), Davidson and Waymouth (1944 *b* and *c*) and Stowell (1946, 1947) have indicated not only that active tumour cells show pentose nucleotides in larger amounts than occur in corresponding healthy cells, but also that a high nucleic-acid content is found in neoplastic tissue, varying in concentration in different regions of malignant growths and often inversely to the amount of fibrous tissue present. It has been shown that pentose nucleotides, either in the free or polymerised state, are among the most common of all cell constituents and are intimately connected with the synthesis of cytoplasmic protein.

Caspersson (1947), in relating the system of protein formation to cell growth, states that this system is intensely active in the cancer cell, where also the increased rate of cell division is related to changes in nucleic-acid synthesis. In such malignant cells the normal endocellular inhibitory mechanism controlling protein formation appears to be largely checked or absent, whereby unrestrained growth of cells takes place. In the healthy cell this system for protein production seems to be checked by certain inhibitors when the amount of material for protein synthesis falls below a definite level. The question arises as to the nature of these endocellular inhibitors.

In the experiments mentioned above it was found that the two purine compounds, the ribose nucleotides adenylic and guanylic acids, exerted a significant inhibitory action on tumour growth, whereas the ribonucleotide, uridylic acid, like its base uracil, had definite growth-promoting properties. Where either of the purine compounds was injected with uridylic acid the accelerated growth of the sarcoma was checked, and the resulting tumour weight conformed to that of the untreated control. The possibility is suggested that the purine

nucleotides may play a considerable part in the inhibitory mechanism in the cell.

Evidence for this possibility is not altogether lacking. The protein formation mechanism indicated by Caspersson includes the intensive production of ribonucleotides in the cytoplasm of the cell external to the nuclear membrane. These nucleotides appear to be derived from substances of a protein nature secreted by the heterochromatin of the nucleus and probably contain a considerable amount of diamino-acids. These diffuse directly from the heterochromatin or via the nucleolus to the nuclear membrane, on the outside of which an active rebuilding of protein, involving ribonucleotides, has been shown to occur. Evidence of these changes in nucleic acid synthesis has been brought forward by Thorell (1944, 1945) and Thorell and Wising (1944) in their descriptions of protein formation in plasma cells in myelomas, and the cycle of events in the development of red and white blood cells.

Further, Spizizen (1943), in his studies on the mechanism of virus multiplication, tested a large number of substrates for the propagation of a bacteriophage, P. He found that in  $8.7 \times 10^{-4}M$  glycine anhydride—which medium appreciably supports virus P multiplication—the percentage increase of the virus in 100 mins. was most marked with yeast nucleic acid. This gave 350 per cent. increase. With guanylic acid as substrate the increase was nil, and it was diminished with adenylic acid. It was suggested that, since yeast nucleic acid contains the four nucleotides in its structure, the increased multiplication of the virus might be due to the action of the two pyrimidines present, and that the purine nucleotides exerted an inhibitory action markedly in contrast with the results obtained with other substrates, except xanthine, which gave only 15 per cent. increase. Such results appear to support the findings in the present experiments, where the pyrimidine nucleotide, uridylic acid, increased tumour growth, but the purine nucleotides inhibited it.

From their studies on various types of nucleic acids and their specific reactions to streptococci and other organisms, Lackman *et al.* (1941) concluded that, in general, nucleic acids precipitate specifically with certain anti-sera, but it was found that the specific reaction could be inhibited by the purine nucleosides, nucleotides and bases. Pyrimidine bases on the other hand showed weak inhibition only and the pentoses and phosphates none at all.

Davidson and Waymouth (1943, 1944a) have pointed out that in rapidly growing tissues there is a high content of cytoplasmic ribonucleotides and that the amounts of simple ribonucleotides are low. It is possible that a disturbance of the normal nucleic-acid metabolism might interrupt complete polymerisation of tetranucleotides and occasion a relative excess of purine or pyrimidine compounds, the former exercising an inhibitory action on cell growth, repair or multiplication, the latter an acceleratory effect. Caspersson and

Santesson in their studies on protein metabolism in tumour cells state that disturbances in the heterochromatic system seem to be a necessary pre-requisite for malignant growth. Such a disturbance in one phase of nucleic acid synthesis might well extend to other anabolic phases, with interruption or suspension of the normal cellular control of the inhibitory mechanism.

Further it is possible that in the rapid polymerisation of nucleotides in sarcomatous growth an excess of diffusible acid-soluble nucleotides escaping polymerisation might be liberated in the extra-cellular fluids and thereby induce the systemic effects previously noted (Parsons, 1945; Parsons *et al.*, 1947). As shown by the experiments detailed above similar tissue changes can be induced in normal mice injected with pentose nucleotides and it is difficult to believe that tissue changes in tumour-bearing mice so nearly identical with those in normal mice treated with these compounds can be due entirely to different causes or combination of causes, more especially when it is remembered that nucleotides are abundantly present in tumour cells.

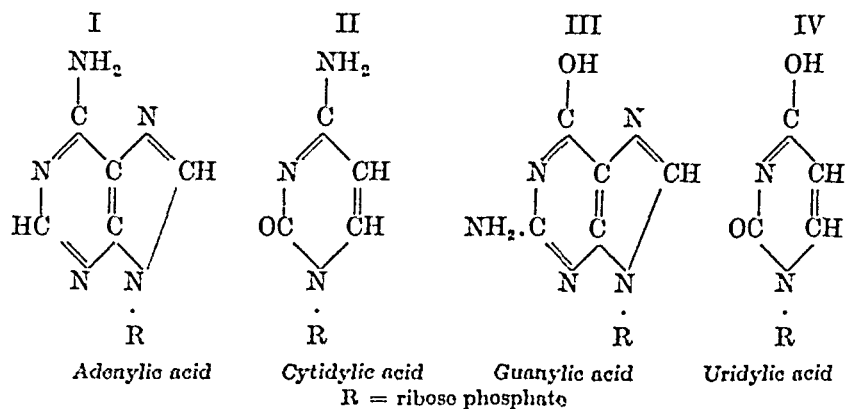
The activity of the nucleotides must necessarily be related to their molecular structure, and the formulæ of their nitrogenous radicals show certain relationships to their biological actions, which may be accidental. This is being investigated.

It has been found that guanylic and uridylic acids exert similar actions on the blood and blood-forming tissues. Both usually cause marked increase in splenic weight and in the number of giant cells; both cause an eosinophilia, which is of longer duration and greater degree in the case of guanylic acid; neither nucleotide increases the number of the other myeloid cells of the blood. Uridylic acid induces a high leucocytosis with absolute increase of lymphocytes, guanylic acid only a moderate leucocytosis with slight increase of lymphocytes. In mice treated with either of these compounds and then injected with adenylic or cytidylic acid, the effect of the latter was frequently masked by the more powerful action of guanylic or uridylic acid. Thus the splenic weight and number of giant cells were generally characteristic of the action of guanylic and uridylic acid, as was also the eosinophilia, and the effect on the blood picture induced by the combined action of the two inhibitors was similar. The action of adenylic or cytidylic acid could usually be detected only by the variations in the polymorph count and the increase of myeloid cells in the blood.

The similarities and differences between the structure of guanylic and uridylic acids can be seen by comparison of the structural formulæ of III and IV (p. 458). In the same way the structure of adenylic acid (I) and cytidylic acid (II) exhibits certain divergences and resemblances and it is to be observed that the biological actions of these compounds on the blood and spleen appear to be similar. Both cause a leucocytosis with increase of immature myeloid cells and a rise in the absolute polymorph values. A rise occurs also in the absolute values



of the lymphocytes, but with wide variations and frequent crossing of the lymphocyte line by the polymorph. Neither nucleotide induces an eosinophilia. Splenic weights tend to be within the normal range and giant cells are diminished in number or are only slightly above normal.



These changes are most marked when the compounds are injected together in normal mice. In all considerations concerning the actions of the nucleotides on tumour-bearing mice the probability of the sarcomatous growth exerting a coincident effect on the tissue changes must be borne in mind, more especially if the tumour be metabolising and liberating ribonucleotides in the body.

It will be noted that amongst the activities of the four nucleotides, the similarity of the effect on the tissues of adenylic and cytidylic acids on the one hand and of guanylic and uridylic acids on the other is not paralleled by similarity between the action of these pairs of compounds on tumour growth. It is thus possible that a relationship between tissue changes and tumour growth may be controlled through the agency of these compounds and that a disturbance in the metabolism of one or more of them may cause a deviation from the normal balance of processes in the cell, the observable effects of which will depend on the excess or deficiency of one or other of the nucleotides. It is also suggested that, by means of the action and interaction between the different nucleotides in the cell and their alternating activities, the balance of normal cell metabolism is maintained and normal responses to stimuli regulated.

Experiments are being undertaken to attempt to correlate the biological reactions and molecular structure.

### SUMMARY

Experiments to test the effect of the pentose nucleotides on tumour growth in mice have shown that the two purine nucleotides, adenylic acid and guanylic acid, exert an inhibitory action, cytidylic acid a negligible effect, and uridylic acid a growth-promoting effect on sarcoma development.

The tissue changes induced by the activity of the nucleotides and related compounds are contrasted and the striking similarity of these changes to those induced by tumour growth in the animal body is emphasised.

We are much indebted to the British Empire Cancer Campaign for grants supporting this research. Our thanks are due to Mr A. J. Reeves for irradiation of the mice, to J. King for the photomicrographs, and to Miss B. Downes for the histological preparations.

His collaborators would record with deep gratitude their memories of the late Professor J. Masson Gulland, F.R.S., to whose genius and large-hearted wisdom this work owes so much.

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OBSERVATIONS ON THE HEPATOTOXIC ACTION  
OF THE CARCINOGEN *P*-DIMETHYLAMINO-  
AZOBENZENE

J. W. ORR and D. E. PRICE

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School of Medicine, University of Leeds*

(PLATES LXXX-LXXXIII)

OF the various azo compounds which are carcinogenic for the liver, the most comprehensive investigations have been made respecting *p*-dimethylaminoazobenzene (DMAB). Its carcinogenic action was at first attributed to a primary growth-stimulating effect on the liver cells, but a majority of subsequent workers appear to prefer the view that the primary changes in the liver are degenerative in nature and are followed by reparative processes out of which cancer ultimately emerges (Orr, 1940, 1946-47; Edwards and White, 1941-42; Opie, 1944, 1946). It is somewhat surprising that but little attempt has been made to study the earliest effects of DMAB. It is not generally realised that this highly toxic substance is capable of killing animals in a few hours. In the present communication we report the results of such experiments, which suggest that these effects are due to hepatotoxic action.

*Material and methods*

Intraperitoneal injections were made of a solution of DMAB in lard (37° C. filtrate). At first the solution contained 3.75 per cent. of DMAB, but this concentration approaches saturation and was troublesome for technical reasons. Most of the later experiments were therefore done with a 2 per cent. solution. Injections in this way give more accurate knowledge of the amount administered than is possible by the oral route, and it is also possible to obtain more reliable information as to the actual amount absorbed. Aqueous solutions are unsuitable because of the low water-solubility of DMAB, and most of the organic solvents are themselves hepatotoxic. Control rats received repeated injections of lard alone over prolonged periods without detectable toxic effect on the liver. Olive oil has also been used as the solvent in a few instances; the results appeared to be identical.

Mixed stock rats were used of an average weight of 210 g. (range 110-300 g.). They were fed on grain (mostly rolled oats, occasionally wheat or maize), bread crusts or rat cake (Thomson, 1936), with water *ad lib*. During the experiment they were kept in separate cages.

The rate of absorption of DMAB was estimated in a separate group of rats which received 150 mg. per kg. body weight and were killed at intervals thereafter. The residue was washed out of the peritoneal cavity with normal saline, reduced to a small volume by evaporation on a sandbath, dried in a desiccator and extracted with ether. The ether extract was made up to a standard volume, to 5 ml. of which were added 0.7 ml. of methylated spirit and 0.2 ml. of hydrochloric acid. This was compared colorimetrically with standard solutions of DMAB similarly treated. The reliability of the method was tested by collecting in a similar way the peritoneal contents of a rat killed immediately beforehand, when 100 per cent. of the injected dye was recovered. The results are shown in the table, and in fig. 1 the amount of DMAB absorbed is plotted against

TABLE

*Rate of absorption of p-dimethylaminoazobenzene after intraperitoneal injections of 150 mg./kg.*

Time in hours	Percentage absorbed
0	0
1/12	26.8
1	61.6
3	65.3
6	76.6
24	95.4
48	98.6
72	98.6
96	97.7
120	98.4
144	98.8
192	98.8

log. time. It will be seen that more than 75 per cent. of the dye is absorbed in 6 hours and 95 per cent. in 24 hours.

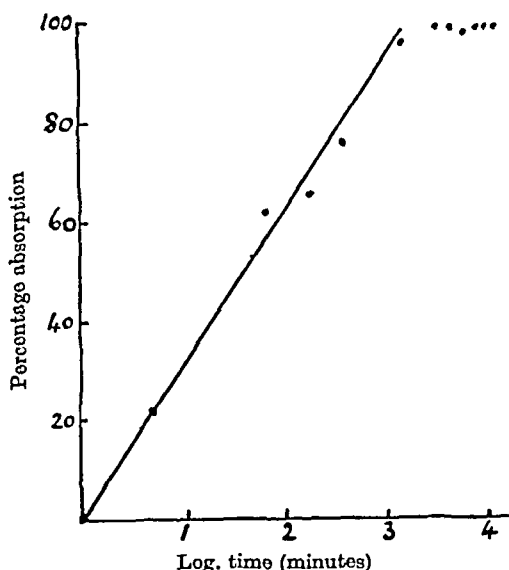


FIG. 1.—Rate of absorption of *p*-dimethylaminoazobenzene after intraperitoneal injections of 150 mg./kg.



amounts of DMAB in single doses were killed and examined, as well as animals in the 150-250 mg./kg. range killed at various times after treatment. In general, histological changes were not striking in animals receiving single doses of less than 100 mg./kg., and somewhat doubtful under 50 mg./kg.

Tissues were fixed in 4 per cent. formaldehyde-saline, embedded in paraffin wax and sections stained with Harris's hæmalum and aqueous conis, Weigert's hæmatoxylin and van Gieson, and other methods as necessary. Frozen sections were stained with Sudan III and hæmatoxylin.

The changes observed in the livers of these rats show a considerable range of variability, both in the severity of individual recognisable processes and in their association with one another. It would therefore be misleading to describe any particular picture as characteristic of the appearance seen after a stated period.

In rats which received very large doses and died within 24 hours there is often no gross abnormality in the liver, apart from congestion. When death is delayed beyond this period, it is generally clear on inspection that the liver is damaged, though the appearances are by no means constant. The liver may be softer than normal, sometimes dark in colour, sometimes pale with a yellowish-brown tinge. The lobular pattern may be exaggerated by congestion of the centres of lobules, or by loss of central substance leading to a slight uniform dimpling following the lobular pattern. On the other hand there may be obscuration of the pattern associated with swelling and rounding of the edges.

With multiple sub-lethal doses there is also variability between exaggeration and obscuration of the pattern in the first place. Later the consistency becomes tougher, and the surface develops a finely granular appearance of lobular distribution. The rate at which this develops varies, but it was observed in one rat after 5 days. Over the period concerned in these experiments, we have found it impossible to correlate visible granularity with the extent of portal proliferative changes of any type: it may be absent with considerable portal proliferation, or present with little. Granularity is generally present, however, after a fortnight's treatment, and subsequent to that there may be adhesions to other viscera, probably the result of local effects due to the method of administration of the DMAB. Ascites has occasionally been seen, but never in any considerable amount.

The weights of these livers have not varied outside normal limits.

### *Histology*

The changes may be divided into those which are essentially degenerative and the various types of reparative proliferation which ensue. The latter are mainly found, of course, in animals receiving multiple doses.

TOXICITY OF *P*-DIMETHYLAMINOAZOBENZENE

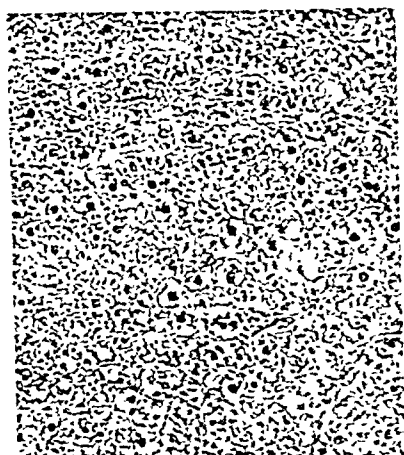


FIG. 3.—Liver of rat which received lard only. Note characteristic vesicular cells due to presence of glycogen.  $\times 150$ .

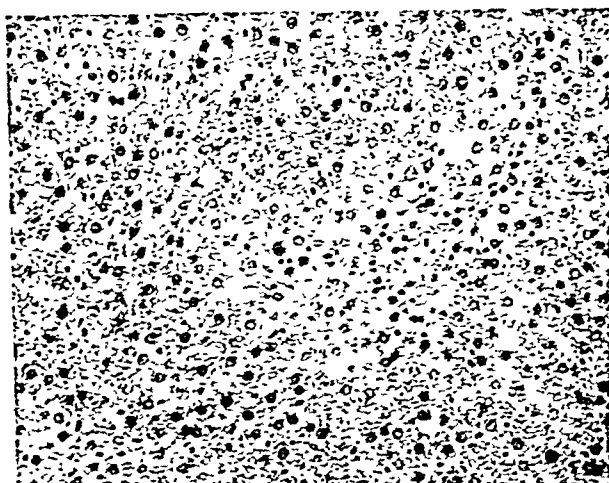


FIG. 4.—DMAB 450 mg./kg. Animal died after 36 hours. Parenchymatous degeneration of liver with fatty vacuolation of cells. Changes most marked in centre of lobules.  $\times 150$ .

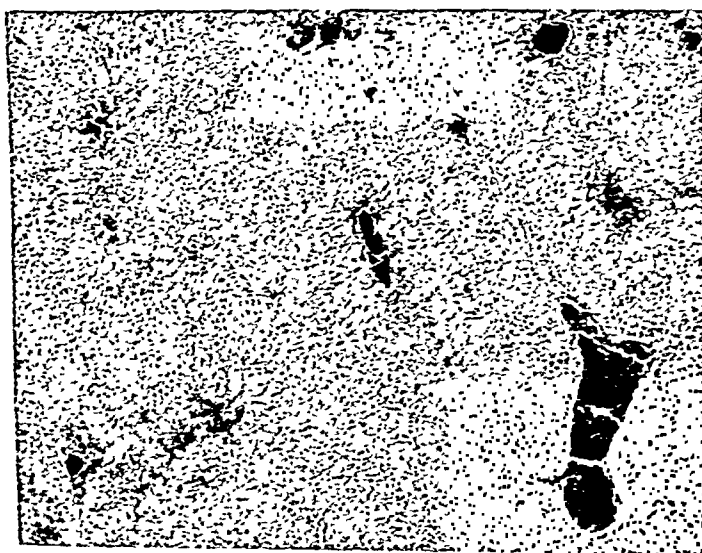


FIG. 5.—DMAB 320 mg./kg. Survived 60 hours. Central necrosis, congestion and hydropic degeneration; parenchymatous degeneration of remainder of lobule.  $\times 50$ .

All sections stained with hæmalum and eosin.



amounts of DMAB in single doses were killed and examined, as well as animals in the 150-250 mg./kg. range killed at various times after treatment. In general, histological changes were not striking in animals receiving single doses of less than 100 mg./kg., and somewhat doubtful under 50 mg./kg.

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TOXICITY OF *P*-DIMETHYLAMINOAZOBENZENE

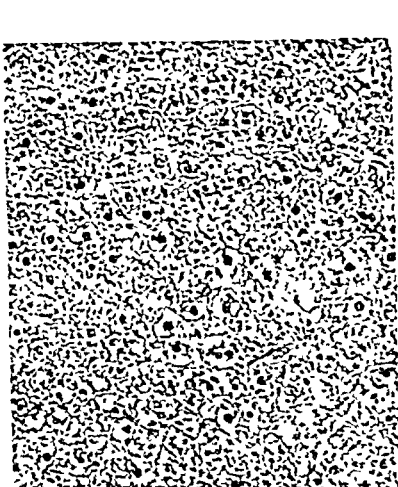


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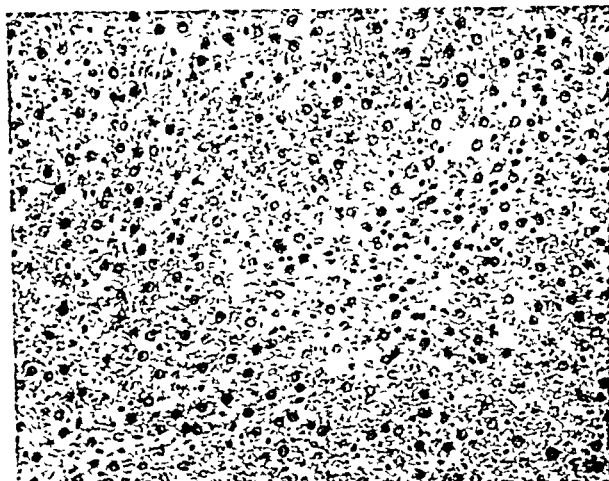


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FIG. 5.—DMAB 320 mg./kg. Survived 60 hours. Central necrosis, congestion and hydropic degeneration; parenchymatous degeneration of remainder of lobule.  $\times 50$ .

All sections stained with hæmalum and eosin.



*Parenchymatous degeneration.* This is the earliest recognisable change and tends to involve the entire hepatic parenchyma. The cytoplasm becomes more deeply stained, and there is loss of the characteristic vesicular appearance due to the presence of glycogen (cf. figs. 3 and 4). It is often the only change detected in rats which die in less than 24 hours.

*Centrolobular degeneration.* This may be detectable in the first 24 hours, but a longer period of survival is generally necessary to make it obvious. It may be limited to increased eosinophilia of the cytoplasm with shrinkage of the cells. With survival for 24 hours there is generally fatty degeneration in the form of fine intracellular droplets in the central cells (fig. 4). A day later hydropic degeneration may be seen, similar to that described by Cameron and Karunaratne (1936) with carbon tetrachloride in the rat (fig. 5). The swollen hydropic cells round the central vein are surrounded by a zone of fatty degeneration. In other instances the predominant central change is one of necrosis, with nuclear pyknosis and karyorrhexis, which may or may not be associated with some leucocytic infiltration or hæmorrhage. We have not seen the empty spaces round the central vein which appear to be characteristic of the carbon tetrachloride reaction during the process of removal of the damaged cells; it would appear that cytolysis is a less active process in the case of DMAB.

*Diffuse degenerative changes.* The clear-cut picture of zonal degeneration is only seen with large (approaching lethal) doses in the acute phases. In the more chronic experiments with multiple doses degeneration is not so massive, and the changes which are seen affect scattered individual cells throughout the lobule, though still more numerous in the internal half. The most obvious histological evidence is in the nuclear alterations described below. On occasion, however, we have had very extensive damage with great loss of parenchyma, approaching the picture of so-called acute yellow atrophy (fig. 6).

*Kupffer cells.* During the stage of degeneration these contain pigment; frequently they are large and loaded with it, sometimes before any manifest evidence of cell destruction is visible in the sections. The pigment is dark brown in colour and consists of fine amorphous granules. It does not stain for iron, nor with fat stains, though scanty sudanophil granules may be found along with it in some of the cells. The most intense pictures of this sort have been seen in the earliest stages, so it appears to be rapidly disposed of; otherwise it would tend to accumulate in the more protracted experiments.

*Nuclear changes.* As mentioned above, pyknosis and karyorrhexis are frequently seen in the central cells. At the periphery of the lobule there are often hyperchromatic nuclei; it is difficult to say whether these are the expression of damage or of a commencing regenerative reaction. In rats which have been treated for 5 days or more, there are nearly always scattered pyknotic cells throughout the lobule, the

number increasing with the duration of the experiment (fig. 7). In some of these the nucleus is fragmented. There are also cells which are devoid of nuclei. After about a week there are usually marked variations in nuclear size, as well as cellular anisocytosis; many giant nuclei are seen, quite outside normal limits: it is, however, difficult to decide whether these changes are to be interpreted as degenerative or reparative. Mitoses have been seen, but they are surprisingly infrequent in this material.

*Inflammatory changes.* There may be slight inflammatory infiltration in association with central degeneration and especially with necrosis. In the multiple dose experiments, the liver parenchyma becomes diffusely infiltrated with scattered round cells having the morphology and staining properties of lymphocytes, but of distinctly larger average size (fig. 7). Leucocytic and lymphocytic infiltration may be found in the portal systems after multiple doses, and often accompanies the portal reparative processes.

*Focal necroses.* These have occasionally been seen, but the evidence suggests that they are of infective origin and not due to the experimental treatment.

*Regeneration after a single dose.* In animals which survived a single dose for a week or more, regeneration of the liver substance and structure appears to be complete, and there are no detectable histological abnormalities. There is no abnormal proliferation.

*Reparative proliferation in the portal systems.* Such changes have only been seen in the multiple dose experiments. The bile ducts or the connective tissue may be implicated (figs. 8-10). There is no obvious correlation; sometimes marked bile duct proliferation is accompanied by but little activity of mesenchymal elements. The proliferating bile ducts do not all remain confined to the portal system itself, but infiltrate between the columns of parenchymal cells in the periphery of the lobules (fig. 9). In general, they show well developed lumina lined by cuboidal epithelium and resemble more closely the normal bile ducts than do the structures seen in an average specimen of human multilobular cirrhosis or subacute necrosis. The connective tissue reaction may take the form of histiocytic proliferation, sometimes with fibroblastic forms, or complexes of new-formed capillary vessels the walls of which consist of double rows of endothelium, with or without lumina. The relative amounts of histiocytic and vascular proliferation vary from animal to animal, and either of them may appear in almost pure form. The process spreads out from the portal system into the periphery of the lobule, and at a later stage outgrowths from different portal systems may meet and divide parts of the liver into unilobular segments. There is very little laying down of collagen in the stages covered by these experiments. The invasion of the periphery of the lobule often results in the cutting off of small islets, or even single cells, of parenchyma, and we believe that the regeneration nodules of liver cells develop from such

TOXICITY OF *P*-DIMETHYLAMINOAZOBENZENE

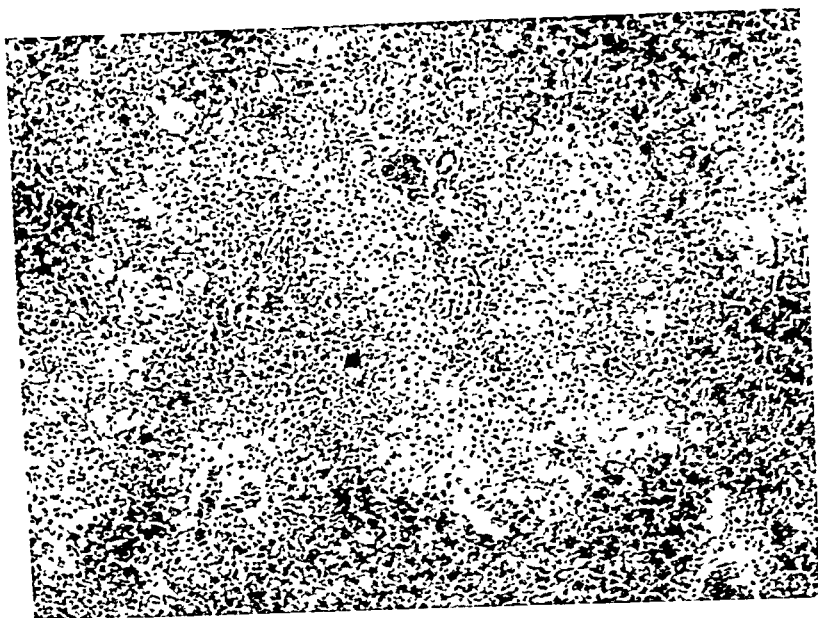


FIG. 6.—DMAB 50 mg./kg. 6 times a week. Survived 37 days. Massive generalised centrilobular necrosis and hæmorrhage, with severe degenerative changes in the much reduced surviving parenchyma.  $\times 100$ .

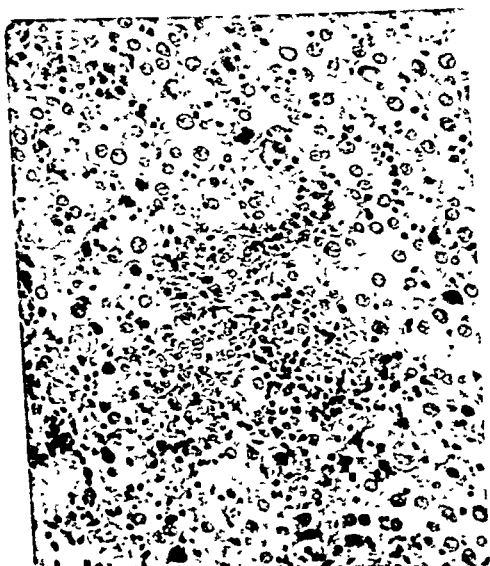


FIG. 7.—DMAB 150 mg./kg. twice a week. Survived 18 days. Inflammatory infiltration of portal systems and parenchyma. Note anisocytosis of parenchyma, nuclear pyknosis and karyorrhexis, and round-celled infiltration.  $\times 180$ .

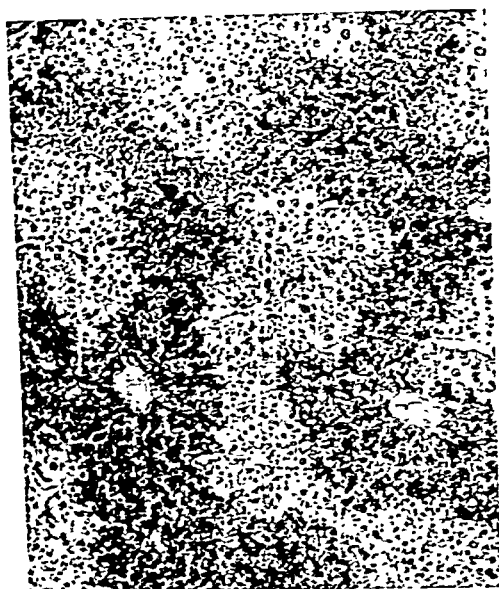


FIG. 8.—DMAB 100 mg./kg. 3 times a week. Survived 19 days. Periportal proliferation, mainly histiocytic and lymphocytic. Marked cellular variation in surviving parenchyma.  $\times 50$ .



sequestrates (fig. 10). Not infrequently the portal proliferation is accompanied by inflammatory cells—polymorphs and lymphocytes—which may sometimes represent the dominant component.

*Regeneration of parenchyma.* It has already been mentioned that after multiple doses changes are found in the parenchyma which cannot with certainty be ascribed to degeneration on the one hand or regeneration on the other. Possibly they are the result of attempted regeneration in the continued presence of the toxic agent. Such changes include anisocytosis, variation in nuclear size and nuclear hyperchromatism. Some of the nuclei are of very large size and irregular shape. Most of the cells contain glycogen. Mitoses are infrequent, which is rather surprising in view of the rapidity with which the parenchyma is being replaced. After about a fortnight's treatment, loss of lobular architecture begins to become evident. This appears to happen in two ways. First, there is disorganisation of the intralobular architecture of individual lobules due to the cellular anisocytosis and to failure to reproduce the characteristic radial arrangement of the columns of hepatic cells. Second, there is the development of regeneration nodules from the sequestrated cells at the periphery of the lobules, the subsequent growth of which is responsible for the profound general loss of architecture and for the coarse granularity when the later cirrhotic stage is reached (figs. 11 and 12).

### Discussion

The present results seem to leave no reasonable doubt that the primary action of DMAB on the liver of the rat is one of damage. The dosage can be adjusted so that there is incontrovertible evidence of hepatic degeneration and necrosis. Examination of other organs has failed to reveal any lesion likely to be lethal in these animals. The only lesion significant in this respect has been parenchymatous degeneration of the kidneys, but this has not been sufficient to produce death and in the multiple dose experiments it has shown no tendency to become progressive. Furthermore, some of the changes elicited with relative rapidity in the present experiments are similar to those we have seen, at a somewhat later date, in feeding experiments in which liver cancer was subsequently obtained in a high percentage of the rats (Orr, 1940). We refer particularly to the diffuse cellular and nuclear changes in the parenchyma. It is probable that in the present experiments the rate of dosage was always somewhat higher than in the feeding experiments, though not greatly so in some of the animals. We estimate that in the feeding experiments the average rate of consumption of DMAB was 30-45 mg./kg. per day. Direct cytological studies by Opie (1946) have also indicated the infliction of early damage on liver cells by DMAB as shown by chromatolysis of cytoplasmic structures containing ribonucleic acid.

Carcinoma of the liver has now been induced by a considerable



number of substances, though those effective in the rat are not necessarily so in the mouse, and vice versa. Many of them are chemically related to DMAB, but there are also unrelated substances such as carbon tetrachloride (in the mouse), chloroform (mouse) and 2-acetylaminofluorene (rat and mouse). With all of these substances there is evidence of damage to the hepatic parenchyma. Cameron and Karunaratne (1936) studied in detail the effects of carbon tetrachloride on the rat's liver. They showed that 24 hours after a single dose of 0.05-0.1 ml. there are well-marked degenerative changes in the centres of the lobules (very similar to our fig. 6), followed in the next few days by autolysis of the damaged cells, mitotic activity of the surviving liver cells, cellular infiltration of the portal systems and a return to normal within a fortnight. With repeated doses, fibrosis develops at the centres of the lobules and in the portal systems, and ultimately the classical picture of multilobular cirrhosis is produced. The principal differences between this reaction and that following DMAB are (i) the absence in the latter of visible cytolysis, (ii) the much slower appearance of collagenous scar tissue and (iii) the appearance of malignant tumours. Qualitatively the reaction is the same; the differences appear to depend on quantitative differences in the speed of various factors. So far as we have been able to discover, carcinoma of the liver has not hitherto been induced with carbon tetrachloride in the rat, but Edwards and Dalton (1942-43) produced hepatomas in 88 per cent. of 263 mice of various strains with this agent. Eschenbrenner and Miller (1945-46) confirmed this result, found that the course of necrosis and repair in the mouse showed no appreciable species difference as compared with the findings of Cameron and Karunaratne for the rat, and came to the conclusion that in all probability the amount of carbon tetrachloride necessary for the induction of liver tumours in the mouse is not necessarily that required for repeated liver necrosis. Eschenbrenner (1943-44) found that the carcinogenic and necrotising effects of chloroform were similar to those of carbon tetrachloride (in female mice; the males died as a result of kidney damage by this agent).

It is sometimes misleading to apply the results with one species to the interpretation of those with another, but in view of the qualitative resemblances between the acute reactions of the rat to carbon tetrachloride and DMAB, it is difficult to avoid the impression that the mechanism of carcinogenesis in the mouse by carbon tetrachloride is related to that in the rat by DMAB. DMAB as a carcinogen has very little activity in mice (Andervont and Edwards, 1942-43), so that in respect of relative potency for species it is the reverse of carbon tetrachloride. The problems created by such apparent contradictions become less baffling if the evolution of the tumour is regarded as the result of regeneration of damaged tissue in the persistent presence of the toxic agent. The fact that cancer can be induced by dosages lower than those required to evoke histologically visible

TOXICITY OF *P*-DIMETHYLAMINOAZOBENZENE



FIG. 9.—DMAB 50 mg./kg. 6 times a week. Survived 32 days. Periportal proliferation, mainly of bile ducts, which infiltrate the receding parenchyma.  $\times 100$ .



FIG. 10.—DMAB 50 mg./kg. 6 times a week. Survived 34 days. Extensive periportal proliferation, mainly vascular and inflammatory. Note the cutting off of small islets of parenchyma by the advancing granulation tissue.  $\times 100$ .



TOXICITY OF *P*-DIMETHYLAMINOAZOBENZENE



FIG. 11.—DMAB 50 mg. kg. 6 times a week. Killed after 36 days. Commencing development of regeneration nodules unrelated to the original lobular architecture.  $\times 30$ .



FIG. 12.—Higher magnification of part of fig. 11, showing active regeneration of parenchyma.  $\times 100$ .



necrosis may only mean that the available methods for the demonstration of the latter are not delicate enough.

### Summary

The toxicity of *p*-dimethylaminoazobenzene for the rat has been investigated. Intraperitoneal injection of amounts exceeding 250 mg./kg. body weight is lethal in less than 3 days; when the dose is less than 150 mg./kg. the animals survive; with intermediate amounts a proportion of the rats die. After intraperitoneal injection of 150 mg./kg. in lard, over 95 per cent. of the drug is absorbed in 24 hours. Animals which survived single doses for more than 4 days, with one exception, did not die as a result of the experiment. Multiple doses of 100 mg./kg. three times a week or of 50 mg./kg. six times a week may be administered with survival of some of the animals for 38 days and over.

The toxic effects appear to be attributable to liver damage. With large doses degenerative changes are most marked at the centres of lobules; with smaller doses or with suitable multiple doses the degenerative changes are more diffuse and less intense. They are followed by reparative proliferation of the hepatic parenchyma, bile ducts and connective tissue. The histological course of these lesions is described.

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## CANINE BETA HÆMOLYTIC STREPTOCOCCI

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EVIDENCE that strains of beta hæmolytic streptococci may cause disease in dogs has been brought forward by Stafseth, Thompson and Neu (1937), Hare and Fry (1938), Stableforth (1938), Minett and Ellis (1940) and Hare (1946). Experimental infection has not, however, been produced in dogs; indeed only one attempt to do this has been recorded (Stafseth, 1940).

The frequency with which beta hæmolytic streptococci can be isolated from apparently normal dogs has been pointed out by Pilot *et al.* (1936) and Garside (personal communication). This suggests that not all strains of beta hæmolytic streptococci are pathogenic for dogs. Of 130 canine strains which were examined culturally and serologically in the present investigation, 44 were obtained from 60 apparently healthy dogs in normal kennels. The others were isolated from dogs with clinical signs and symptoms suggestive of streptococcal infections, from autopsies of puppies that had died of a neo-natal septicæmia, or from contacts of the dogs in the infected kennels.

## METHODS

Swabs were taken from the urethra of dogs, the vagina of bitches and the tonsils of both sexes, but it was found that these swabs were valueless unless particular care was taken to exclude contaminating organisms. Three per cent. lysol was usually satisfactory for cleaning the vulva without having to clip the hair from the part. As a rule, cultures were made promptly on 5 per cent. horse-blood agar, although it was found that streptococci remained viable for at least 24 hours on swabs sent through the post. Tests for soluble hæmolysin were carried out by the method of Hare and Colebrook (1934), but the results were read at various times up to 4 hours. Polysaccharide extracts for grouping were made by Lancefield's (1933) or Fuller's (1938) method. The presence of group precipitin was detected by layering the extracts on the specific group antisera in capillary tubes.

Heavy suspensions of heat-killed cocci were inoculated into rabbits for the production of antisera. To prepare group-M antisera prolonged immunisation was needed. Type precipitins invariably appeared before group precipitins, but finally 5 reasonably reliable sera were made for this group, one serum with SHC 194, a strain supplied by Dr R. M. Fry, and the remainder with 4 strains of the present series. After absorption the same sera were used to type the strains within group M by the slide-agglutination method of Griffith (1927, 1934). A number of strains within groups G, L and M were tested for their bactericidal activities on the lines indicated by previous workers.



## OBSERVATIONS

*Cultural characters*

Three main cultural types were recognisable on blood agar and chocolate agar. These were common to strains of all 3 serological groups of the present series. The first type of colony was smooth, opaque and homogenous—the typical “glossy” colony; the second was a “matt” colony, semi-transparent or translucent, with a variable surface, contour and margin; the third had a glossy centre and a matt periphery, with an appearance rather like that of a poached egg. On subculture from each of the two zones, it was possible to produce a colony in which one or other zone predominated. Some individual strains yielded more than one type of colony. The distribution of these cultural types among 59 strains is shown in table I.

TABLE I

*Distribution of colonial types among 59 strains of hæmolytic streptococci of groups G, L and M*

Group	No of strains		
	Glossy	Matt	Mixed
G	8	0	17
L	3	2	7
M	4	6	12

The only two “R” variants showed large, flat, translucent, granular colonies with a very irregular edge; both belonged to group M. “Watery” or “pseudo-glossy” colonies were not observed. The colonies of 16 strains, 13 of which belonged to group G, were of the coherent variety; other colonies could be broken up easily or were “mushy” in consistency. On the whole, the number of strains producing glossy colonies increased in the chocolate agar medium.

*Hæmolysis*

All strains showed beta hæmolysis on primary isolation on plates under aerobic conditions, except one group-G strain from the milk of a Corgi bitch, which had been treated for mastitis with sulphonamides and penicillin. This strain showed typical alpha hæmolysis on first isolation but gave beta hæmolysis under anaerobic cultivation; it also yielded soluble hæmolysin.

The ratio, hæmolytic zone : colony diameter, was greatest with the group-M strains, with some of which it was as much as 16 to 1. Colony size ranged from pin-point size (3 strains) to a diameter of 1 mm., except for the two rough strains, which reached a diameter of 2 mm.

The results of the tests for soluble hæmolysin with 118 of the 130 strains are shown in table II. In some, particularly in group-M strains, the hæmolysin was weak and acted slowly.

TABLE II

*Number of strains with soluble hæmolysin among 118 strains of canine streptococci of groups G, L and M*

Group	No. of strains tested for soluble hæmolysin		
	Positive	Negative	Total
G	45	13	58
L	1	11	12
M	26	22	48

The rough variant of one group-M strain produced soluble hæmolysin and its glossy variant did not.

### *Growth in broth*

Most of the strains grew well in glucose broth and produced a deposit with a clear supernatant fluid. With most, the deposit was flocculent, but with some it was granular. A few group-M strains produced a uniformly turbid growth.

### *Biochemical activities*

**Group G.** Among 24 strains examined the final pH of glucose-broth cultures ranged from 4.5 to 4.9. Most strains fermented lactose, sucrose, maltose, salicin and trehalose, although eight failed to ferment trehalose. With seven exceptions they did not ferment sorbitol, and eight failed to hydrolyse sodium hippurate. None attacked glycerol, inulin, raffinose or mannitol.

**Group L.** Only six strains were examined. All fermented lactose, sucrose, salicin, maltose and trehalose. Their action on sorbitol and glycerol was variable. All failed to hydrolyse sodium hippurate. The final pH in glucose-broth cultures ranged from 4.7 to 5.2.

**Group M.** The 28 strains examined included the seven type strains kindly supplied by Dr R. M. Fry. They fermented lactose, saccharose, maltose, and, with three exceptions, trehalose. Their action on salicin and glycerol was variable. All failed to ferment sorbitol and to hydrolyse sodium hippurate. The final pH in glucose-broth cultures ranged from 4.6 to 6.9.

*Serology*

*Grouping.* It was found possible to group 118 of the 130 strains. Four strains did not react with any of the grouping sera, and eight of those collected early in the work died in culture before the antisera were available. From the anomalous results shown in table III it is

TABLE III

*Anomalous results with precipitin tests of group-M streptococci*

Antisera to strains	Extracts from strains										Row
	36	50	55	56	SHC 194	SHC 220	SHC 2-5	Sal	SHC 205	SHC 252	
36	+	-	-	+	-	-	-	+	+	+	-
50	-	+	+	+	-	-	-	+	-	+	+
55	+	+	+	+	+	+	-	+	-	+	+
56	-	+	+	+	-	-	+	+	+	+	-
SHC 194	-	-	+	+	+	-	+	+	+	+	+

obvious that a range of group antisera must be available in order to group all strains within group M. It is probable that optimal conditions are needed for a positive result when the extract and the group antiserum are brought together in a ring test, otherwise false negatives will be recorded. A range of extract dilutions was tried in negative tests, but this did not increase the number of positive results. Similar trouble was experienced with a number of group-G strains. Fry's observation, quoted by Wilson and Miles (1946), that formamide extraction destroys the specific polysaccharide of group-M strains was confirmed.

*Typing of group-M strains.* Agglutinin for strain 55 was completely removed from the serum of strain SHC 194 by absorption with strain 55, but the absorbed serum still gave a clear-cut positive slide-agglutination test with SHC 194. A similar result was obtained by absorbing the serum against strain 55 with SHC 194. With these two absorbed sera as typing sera, two serological types were distinguished among group-M strains. Type 1 is represented by SHC 194 and five of the other group-M strains supplied by Dr R. M. Fry, together with six strains isolated during this work. Nine other strains isolated during the present investigation, including strain 55, belong to type 2.

For these tests the strains were grown in 5 per cent. serum broth and were used within 24 hours; older suspensions were sometimes found to give false positive results. Several strains failed to provide suspensions stable enough for the test, even when grown in trypsinised broth. Three strains could not be typed because they gave negative results with both type sera. They may represent an additional type or types, or they may lack a type-specific antigen. Dr Fry's strain

SHC 220 gave a doubtful positive reaction at a titre of 1 in 8 with crude sera, which invalidated negative results with the two type sera in a dilution of 1 in 12.

### *Distribution of strains*

Kennels with clinical cases of infection yielded 54 strains of group G (12 from contacts without evidence of infection), 12 of group L and 11 of group M. The distribution of the various groups among dogs in uninfected kennels is shown in table IV.

TABLE IV

*Incidence and distribution of hæmolytic streptococci of groups G, L and M among dogs in uninfected kennels*

Kennel	No. of dogs	No. of strains	Groups		
			G	L	M
1	6	5	1	0	4
2	5	3	0	0	3
3	4	3	0	0	3
4	10	6	1	0	5
5	6	6	0	0	6
6	9	7	0	0	7
7	16	11	1	0	10
8	6	3	2	0	1
Total . .	62	44	5	0	39

### *Neonatal deaths*

Of 13 puppies that died soon after birth, nine gave group-G strains and 4 gave group-L strains. Unfortunately it was not always possible to obtain material from both puppies and bitch. In six cases where material from both was examined, strains of the same group were isolated from the vagina of the bitch, and sometimes from her tonsils, and from the heart blood and internal organs of the puppies.

### DISCUSSION

The findings confirm the prevalence of beta hæmolytic streptococci among both healthy and clinically infected dogs. The incidence of hæmolytic streptococci in this series of normal dogs in uninfected kennels was 70 per cent. (table IV); but with very few exceptions these strains belonged to group M. Only five healthy dogs among the 44 with hæmolytic streptococci in these kennels were carriers of group-G strains. Strains isolated from cases of clinical disease, on the other hand, belonged to groups G and L, with group-G strains predominating; and a small number of apparently healthy contacts in the infected kennels were found to be carrying group-G strains. It is probable, therefore, that group-M strains are without pathological significance, because in this work a group-M strain was never found

alone in a clinical case of disease. If this is true, there is an obvious advantage in grouping canine strains, particularly if the veterinary surgeon desires to deal with whelping troubles by applying prophylactic or therapeutic measures (drugs or vaccines), or to eradicate the carrier state in any dog. The distinction between the different strains can only be made serologically, as their biochemical activities are not sufficiently distinctive to separate them.

### SUMMARY

1. One-hundred-and-thirty canine strains of beta hæmolytic streptococci were investigated culturally and serologically; 58 were examined biochemically. The streptococci were isolated from cases of presumed streptococcal infection and their contacts and from normal dogs in kennels where there had been no infection.

2. The prevalence of beta hæmolytic streptococci among dogs was confirmed by their isolation from 70 per cent. of healthy dogs in uninfected kennels. Among these strains 39 of 44 belonged to group M, and it is suggested that these are of no pathological significance. The strains within this group were found to belong to at least two serological types by agglutinin-absorption tests.

3. The strains isolated from clinical cases of disease and from a small number of contact carriers belonged to groups G and L, 54 strains being in group G and 12 in group L.

4. It is considered that, in view of the prevalence of beta hæmolytic streptococci in dogs generally, grouping is an important prelude to effective prophylaxis and therapy in canine streptococcal infections.

I wish to thank Dr R. M. Fry, Dr P. M. F. Shattock, Dr A. W. Stableforth and Mr J. S. Garside for stock cultures and sera and for helpful advice, Mr E. Weissberg, who helped to supply much of the material, and Miss V. Cracknell and Miss E. Murdoch for valuable technical assistance.

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# IDIOPATHIC ("ISOLATED") MYOCARDITIS IN INFANCY

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(PLATE LXXXIV)

"ISOLATED" myocarditis was originally described as acute interstitial myocarditis by Fiedler (1899, cited by Šikl, 1935-36) and the heart lesion in his cases was studied by Schmorl (cited by Šikl). The condition has received some attention in the American literature but practically none in the British. It is a special, non-specific lesion characterised clinically by progressive myocardial failure, often terminating in sudden death, and at autopsy by cardiac enlargement and the frequent presence of mural thrombi. There are two histological types, (1) a focal granulomatous lesion which must be carefully distinguished from an atypical rheumatic or pyæmic effect, and (2) a diffuse form, characterised by cellular infiltration, myonecrosis and the occasional formation of muscle giant cells, of which an eosinophilic variety is an unnecessary subdivision.

This paper is concerned with an example of the diffuse form, of which Saphir (1941) lists 15 in a review of 240 cases of myocarditis. It is very uncommon in children. Smith and Stephens (1938) record two cases aged 10 and 13 months, Singer (1932) two aged 6 and 13 months, in the younger of which diphtheria was not entirely excluded, Maslow and Lederer (1933) one aged 21 months and Lindberg (1938) one of 11 months which, however, followed pertussis.

## Case report

*Clinical summary.* A male child, the seventh offspring of healthy parents. Siblings well. Birth weight 6 lb. 6 oz. Breast-fed for 8 months and then put on a mixed diet. Circumcision at 8 months was followed by hæmorrhage but this was not sufficient to cause anxiety. At the age of 10 months vomiting and dyspnœa commenced and the infant was admitted to hospital. The temperature was normal, pulse 130 and respirations rapid—up to 60 per minute. Cyanotic attacks were relieved by oxygen but eventually cyanosis was evident at all times and death occurred at the end of the 11th month. No other physical signs were observed but radiologically the heart was shown to be enlarged.

*Laboratory investigations.* Throat swabs yielded *Str. viridans* and *N. catarrhalis*. Two blood counts were made. The first, 24 days before death,

showed Hb. 50 per cent., red cells 3·3 million, colour index 0·75, leucocytes 12,350. Differential count: polymorphs 46, small lymphocytes 29, large lymphocytes 24, and monocytes 1 per cent. The second, four days before death showed Hb. 50 per cent., red cells 3·35 million, colour index 0·74, leucocytes 13,150. Differential count: polymorphs 60, eosinophils 1, small lymphocytes 16, large lymphocytes 20 and monocytes 3 per cent.

*Autopsy.* The positive findings may be summarised as cardiac enlargement with venous congestion of lungs and liver and, to a lesser degree, of the other organs. The pericardium contained 3 ml. of clear fluid. The heart as a whole was enlarged, weighing 83 g., and, *in situ*, the right side was greatly distended. All the valve cusps were normal and the foramen ovale and ductus arteriosus were closed. The external and cut surfaces of the myocardium were pinkish-brown, with a lighter pattern of yellow streaks superimposed in an irregular manner upon the ventricles. Both ventricles were hypertrophied, the right to a maximum thickness of 2·5 mm., while the left varied from 7 mm. at the base to 5 mm. at the apex. At the apex of the left ventricle there was a fragment of ante-mortem mural thrombus overlying an area of myonecrosis and early fibrosis. The coronary arteries were normal. A careful but fruitless search was made for any possible focus of infection.

Portions for histology were taken from both ventricles, both auricles, the interventricular septum, including several segments of the anterior descending branch of the left coronary artery, the aorta and pulmonary artery and the following viscera: brain, lung, alimentary tract, liver, spleen, kidney, lymphoid tissue, marrow, adrenal, pancreas and pituitary. Sections were stained with hæmatoxylin and eosin, iron hæmatoxylin and van Gieson, phosphotungstic acid-hæmatoxylin and Wilder's reticulin impregnation, and by Sudan III for fat.

*Histology.* The heart alone requires detailed description. The other organs show only congestion and œdema. The lesion in the myocardium is an interstitial cellular infiltration (fig. 1), predominantly lymphocytic but including plasma cells, histiocytes and a few polymorphs in and near the areas of myonecrosis. The cells appear singly and in small groups in intimate relation to the capillaries and where the lesion is more pronounced they form dense groups, with interlacing strands in which the muscle fibres are distorted and eventually destroyed (fig. 2). The muscle fibres in relation to the cellular infiltration show progressive stages of dissolution; loss of transverse striation, gradual disappearance of the central myogial fibrils and finally complete lysis, leaving a pallid nucleus surrounded by inflammatory cells. The muscle fibres not in close relation to the areas of infiltration show no signs of degeneration and in this respect the lesion differs from that of the myocarditis associated with Friedreich's ataxia (Russell, 1946). The process is most marked in the left ventricle, including the interventricular septum, but the whole

## IDIOPATHIC MYOCARDITIS

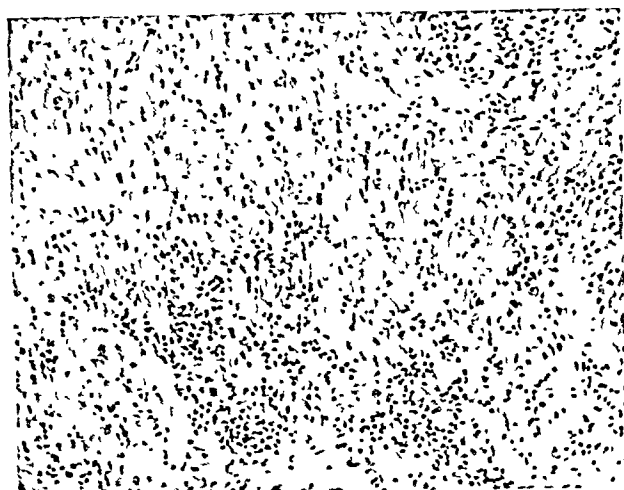


FIG. 1.—Apex of left ventricle showing interstitial cellular infiltration and myonecrosis. Hæmatoxylin and eosin.  $\times 125$ .

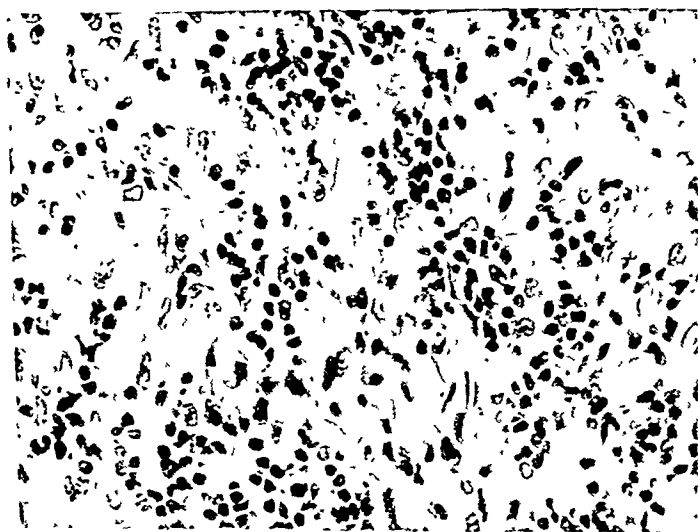


FIG. 2.—Interventricular septum. Degeneration of muscle fibres in cellular areas and hypertrophy of remainder. Hæmatoxylin and eosin.  $\times 380$ .



FIG. 3.—Left ventricle from a case of asthma. Sparse cellular infiltration, hypertrophy and fibrosis. Hæmatoxylin and eosin.  $\times 136$



fibres in the meshes. Franz stressed the importance of primary muscle damage, due either to epinephrine or to hypersensitivity to it. I have recently been able to study the heart of an asthmatic woman who had been treated with epinephrine. The left ventricle was slightly hypertrophied, with grey lines of fibrosis visible. Histologically it presents a coarse reticular fibrosis (fig. 3) with a sparse interstitial scattering of eosinophils, lymphocytes and occasional plasma cells. There is no evidence of recent myonecrosis, though the woman died in a most acute phase of the disease.

In the case of the allergic adult it is difficult to assess what is due to the condition and what may be due to the treatment, but from what we know of the allergic reaction it is reasonable to postulate that the lesion develops in connective tissue and affects the parenchyma of the particular organ secondarily. The fact of secondary affection of the parenchyma is clear in the case of myocarditis reported. The chronic lesion of the asthmatic heart is equivocal but the experimental evidence suggests that epinephrine alone is not a causative agent.

*Virus infection.* Helwig and Schmidt (1945) isolated a virus from a group of anthropoid apes dying of interstitial myocarditis and reproduced the disease in mice. In a further paper (Schmidt, 1948) the properties of the virus are described. It produces myocarditis and encephalitis in mice and hamsters and myocarditis in guinea-pigs. The heart lesion varied from slight perivascular lymphocytic aggregates to advanced myonecrosis and polymorph infiltration. The agent was potent and specific by the intravenous, intraperitoneal, subcutaneous, intracranial and intranasal routes. The lesion closely resembles that of non-specific (idiopathic) myocarditis, but neutralisation tests on human contacts suggested that this particular virus was probably non-pathogenic to man and so far there are no clinical data to indicate the existence of human infectious myocarditis.

So far all attempts at explanation have led to the introduction of further unknown quantities and until a new approach to the subject can be developed by the study of many hearts with a careful clinical correlation it is reasonable to regard the condition as a special connective tissue response to an agent not necessarily organic. This variety of myocarditis would thus be brought into line with polyarteritis nodosa, and in this connection it may be mentioned that polyarteritis nodosa affecting small vessels closely resembles an acute interstitial inflammation. Variations in the histology are determined by the size of the vessels involved and the relative proportions of parenchymatous tissue and stroma. The interstitial cells of myocarditis may well be derived *in situ* from undifferentiated mesenchyme.

### *Summary*

myocarditis of unknown aetiology and uncomplicated  
r is described. Apart from the rarity of the

condition in infants it is of interest in that it can be studied without having to take into consideration the many ailments that beset advancing age. The distribution of the lesion is noted in some detail and it is suggested that the condition is a true interstitial myocarditis determined by an unknown stimulus affecting the capillaries and connective tissues of the heart.

I am indebted to Dr R. V. Dent for the clinical notes, to Dr A. B. Bratton for helpful criticism and to Mr G. W. Moore for the photomicrographs.

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## LIPOSARCOMA ARISING IN A SIMPLE LIPOMA

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(PLATES LXXXV-LXXXVII)

LIPOMA is a common, liposarcoma a relatively rare tumour. Malignancy arising in a pre-existing simple lipoma is a great rarity, of which the case here reported is a particularly fine example.

## Case report

*Clinical history*

The patient, a woman aged 69, complained of a massive swelling on the inner aspect of the left thigh. There had never been any pain and the only reason why she wanted it removed was on account of its large size and awkward position rendering defaecation and micturition difficult. About 7 or 8 years ago she first noticed the swelling, which gradually increased in size. Her doctor dismissed it as a "fatty tumour". A year or two before its appearance she had been involved in a motor-cycle accident, and to this she attributed the tumour. There was no serious injury, the upper outer part of her left thigh merely sustaining a "knock". She had always had fat thighs, and after a time the tumour stopped growing and she ceased to notice it. During the last few months, however, the tumour had again increased in size and had become a nuisance. The patient felt well generally.

Physical examination revealed a large swelling on the medial aspect of the left thigh, extending upwards as far as the pubis and down nearly to the knee. The surface was irregular and felt shotty anteriorly, but was smoother, harder and more sharply defined posteriorly. The overlying skin was slightly reddened and warm, and showed veins coursing over the surface. The tumour was attached to the adductor muscles and was rendered immobile and more easily defined by contraction of these muscles, being freely movable when they were relaxed.

X-ray examination of the left thigh showed a large soft-tissue swelling which appeared to have a capsule of fat, this tissue being radiolucent. Amorphous calcareous deposits were present, and the tumour appeared to be quite separate from the bone.

At operation on 12th February 1948 the tumour, which was removed by Mr George Armitage, was enucleated from the midst of the adductor group of muscles. It extended backwards to the ischiorectal fossa. The anterior part appeared to be lipomatous, but the posterior part was regarded as undoubtedly malignant.

At the time of writing, nine months after operation, the patient is in good health, with no sign of recurrence.

*Morbid anatomy*

This large tumour, weighing 6 lb., consisted of two main portions each some 5 or 6 in. in diameter and united by a thick fibro-fatty band. One tumour was grossly lobulated, well encapsulated, yellow and translucent—a typical benign lipoma; the other was firmer, whiter and more opaque in its non-necrotic portion, roughly spherical and only slightly nodular. The surface of this portion was rather shaggy in places, due to attached fibrous tissue and muscle which had been removed with the growth. Several fairly large lobules of adipose tissue were also attached. The colour was greyish white, with scattered dark-coloured areas. The tumour had penetrated the capsule in a few places, forming button-like outgrowths. The band connecting the two main masses was composed of simple adipose tissue and fibrous bands. On section (fig. 1) the lobulated tumour was homogeneous and yellow throughout—a typical benign lipoma. The other part of the tumour had an obviously malignant appearance on section, consisting of dense white opaque growth, with myxoid and hæmorrhagic areas and scattered cavities filled with fibrin and blood clot. The tumour was enclosed within a capsule of varying thickness which contained many tiny foci of calcification.

*Histology*

The simple lipomatous portion of the tumour, the adipose tissue in the connecting band and the lobules attached to the periphery of the malignant portion are formed of mature fat cells and show no sign of malignancy. The malignant portion of the tumour is sarcomatous, with extensive cellular areas of undifferentiated round and spindle cells (figs. 2-4), scattered myxoid areas (fig. 5) and areas of necrosis; fibrin and blood clot are present in large amount. For the most part the round and spindle cells have granular cytoplasm, but many of them show vacuolation (fig. 2), from tiny vacuoles, single or more often multiple, to large single vacuoles giving the cell a signet-ring appearance (figs. 3 and 4). Some of the latter have the appearance of mature fat cells, but the majority have large, hyperchromatic and often bizarre nuclei (fig. 4). Occasional cells show multiple large vacuoles (fig. 3). The nuclei vary greatly in size and shape and mitotic figures are numerous, with occasional multipolar types. The vacuolated cells are either separate or arranged in groups (fig. 2); in some areas these groups are large and numerous. Frozen sections stained with Scharlach R show that in a large proportion of the tumour cells the cytoplasm takes the stain in the form of tiny granules. The content of the larger vacuoles also takes the fat stain. This is most evident in the degenerate areas but also occurs in areas where the tumour cells are healthy. A striking feature of the tumour is the presence of deeply eosinophilic intra-cytoplasmic globoid bodies

LIPOSARCOMA ARISING IN SIMPLE LIPOMA



FIG. 1.—Tumour on section, showing benign lipomatous portion on left, liposarcoma on right.  $\times 3$ .

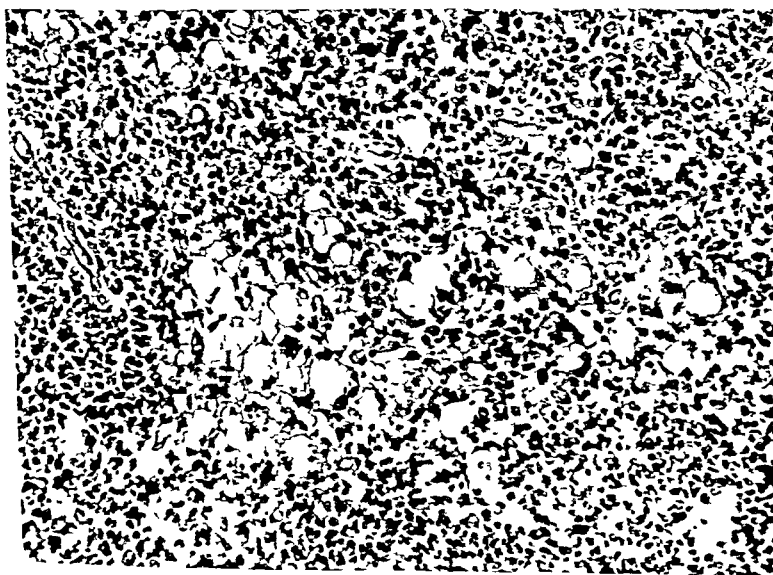


FIG. 2.—Cellular round-cell area showing groups of vacuolated (fat) cells.  $\times 150$ .



LIPOSARCOMA ARISING IN SIMPLE LIPOMA

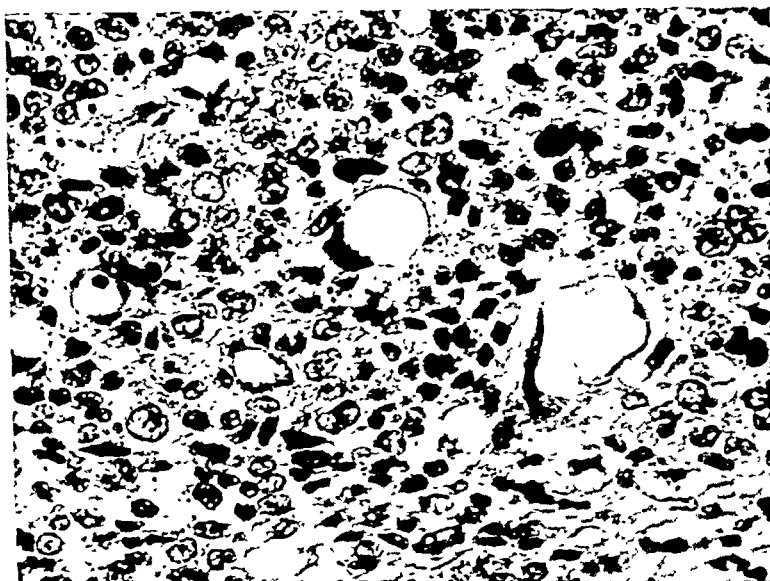


FIG. 3.—Malignant lipoblasts, one with multiple large vacuoles.  $\times 340$ .

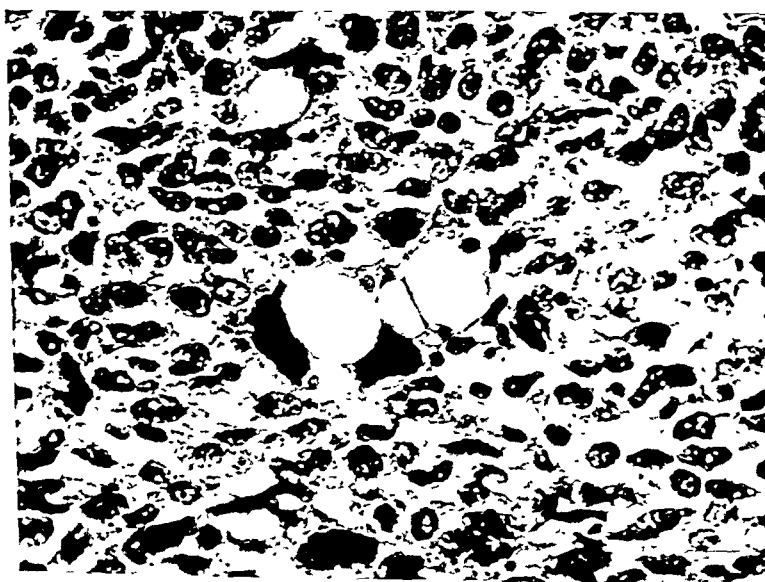


FIG. 4.—Vacuolated giant cells with bizarre hyperchromatic nuclei and a mitotic figure.  $\times 420$ .





(fig. 6), ranging in size up to 10 or 12  $\mu$ . Sometimes they appear to be situated within the fat vacuoles. The resemblance to hyaline droplet degeneration is remarkable. These bodies are particularly common in the more degenerate parts, but not invariably. In the myxoid areas the relatively sparse spindle and stellate cells are united by fine cytoplasmic, faintly eosinophilic threads. In the more cellular areas the matrix is more eosinophilic and shows numerous small vacuoles.

#### COMMENTARY

It is well known that while simple lipomas are very common, liposarcomas are rare, and authentic examples of liposarcoma developing in a pre-existing lipoma are rare indeed. As Willis (1948) states, the term "liposarcoma" should be restricted to tumours composed of the characteristic cell, the lipoblast. The cells forming the malignant portion of the tumour here reported were typical lipoblasts, the more immature forms with granular cytoplasm and early fat vacuoles, the older containing large, single or multiple vacuoles, each filled with a single droplet of neutral fat.

Liposarcoma usually occurs in middle-aged people, the sex incidence being more or less equal, and is most common in the soft tissues of the lower extremity, particularly intra- or intermuscular. On the whole, trauma appears to play a minor part in causation. It was not recorded in any of the cases reported by Stout (1944), but it has been reported by other authors. As regards rate of growth, the tumour may grow slowly over a period or growth may be extremely rapid. Recurrence and metastasis are the rule in spite of the apparent encapsulation of the growth. In the present instance recurrence is to be anticipated, in view of the penetration of the capsule and the anaplastic nature of the growth. In Geschickter's (1934) series of twelve cases the tumour recurred after excision in all but one, and that case had only been recently observed. No case was reported "cured".

It is most probable that the great majority of lipoblastic tumours are malignant from the beginning, but cases of malignant change in a pre-existing lipoma do occur. How frequently this happens it is impossible to say in tumours formed of a mixture of adult fat cells and lipoblasts, since this condition may or may not have existed from the beginning. Few cases appear to have been recorded where a liposarcoma was associated with a simple lipoma as in the case here described. The only case which I have been able to find in the literature bearing any resemblance to the present instance is one reported by Stout. Case 21 in his series was a retroperitoneal liposarcoma which was completely surrounded by simple lipomatous tissue. The only details given are that the patient was a male aged 86, that the tumour was retroperitoneal (above the bladder) and of seven months' duration before diagnosis, its size 8×8 cm. The central

malignant portion was of poorly differentiated myxoid type and was situated as stated within a larger simple lipoma.

Schiller (1918) and Katz (1928) are quoted by Stout, but Schiller's two cases were a fibrosarcoma and spindle-cell sarcoma respectively, each arising in a simple lipoma. Of Katz's two cases, one was a retroperitoneal lipoma of large size which was removed surgically and, it was thought, completely. It was described histologically as a fibrolipoma. Eighteen months later the tumour recurred, but there is no mention of further surgical treatment or of histological examination. The second case was also retroperitoneal, in a woman of 39. At operation a lipoma 18 lb. in weight was apparently completely removed. Two years later she had an operation for recurrence. No mention is made of the histology of either tumour. Three years later still the patient was admitted to hospital in a wasted condition with a second recurrence, which at operation could not be completely removed. Histologically the tumour was now said to be a liposarcoma.

Geschickter reported 18 cases of fibrous or embryonic lipoma, 9 of which showed one or more recurrences and 4 of these recurrent growths subsequently underwent malignant change. Although liposarcoma secondary to benign lipoma is mentioned in Geschickter's classification of "lipoid" tumours, no typical example is reported.

In Shaw's (1936) case and in that of Seidelin (1932) a few lobules of yellow adipose tissue adhered to the capsule of a liposarcoma.

Robertson (1916-17) in a survey of 51 cases of "lipoma myxomatodes" reported prior to 1916, quotes 2 cases of interest. Walser, in 1881, reported a retroperitoneal "myxoma-lipomatodes", the larger portion of which was lipomatous, and Rafin, in 1885, a lipoma becoming myxomatous. Both were stated to be non-malignant. In the case reported by Robertson the tumour showed an area which had the appearance of adipose tissue to the naked eye, but histologically was almost identical with the part containing mucoid fluid alone.

Adair, Pack and Farrior (1932) reported a case in which "after ten years of steady growth a lipoma originally benign underwent malignant change". The excised tumour, however, showed no sign of simple lipomatous tissue and the case is therefore open to doubt.

#### SUMMARY

The case described is one of tumour of the thigh, quiescent for 7 or 8 years and then, recently, increasing in size. On excision it was found to be a liposarcoma arising in a simple lipoma. A search has been made in the literature for comparable examples, and only then was the rarity of the condition really appreciated.

I am indebted to Mr George Armitage for permission to publish the case and to Prof. M. J. Stewart for his interest and advice.

LIPOSARCOMA ARISING IN SIMPLE LIPOMA

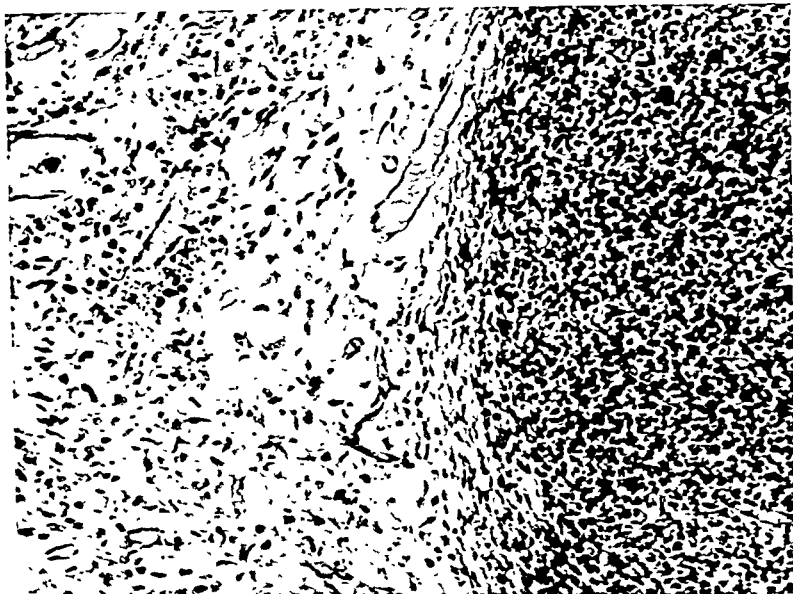


FIG. 5.—Showing contrast between cellular round- and spindle-cell area and myxoid area.  $\times 110$ .

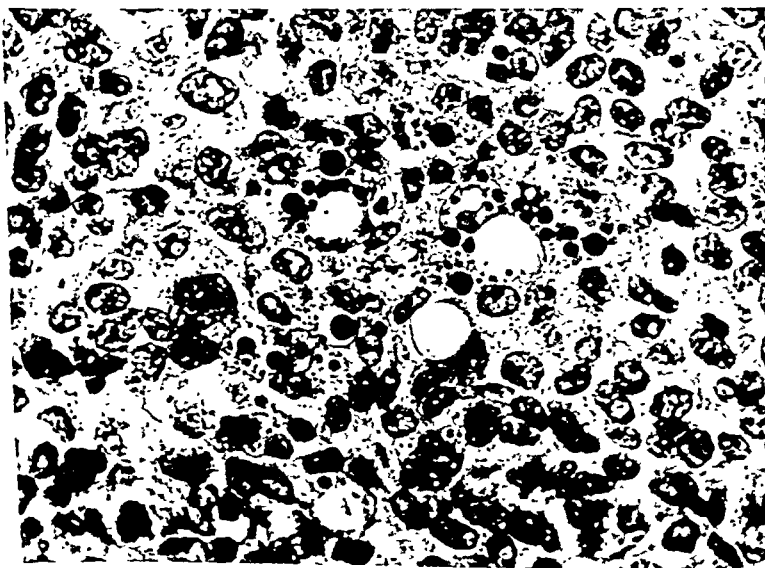


FIG. 6.—Eosinophilic globoid bodies both in the cytoplasm and in the fat vacuoles.  $\times 420$ .



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$$616-022 \cdot 76 \cdot 809 \cdot 72 : \begin{cases} 616-091 \cdot 818+ \cdot 155 \cdot 35 \\ 616 \cdot 13-002 \end{cases}$$

## DISSEMINATED FOCAL NECROSIS WITH EOSINOPHILIA AND ARTERITIS IN A CASE OF ASTHMA (? LOEFFLER'S SYNDROME)

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(PLATES LXXXVIII AND LXXXIX)

IN view of recent interest in conditions where asthmatical symptoms are associated with eosinophilia and transitory pulmonary lesions, and the rarity of post-mortem observations on such cases, it is considered justifiable to record a single instance in which at necropsy widely disseminated lesions of an unusual type were found.

### Case report

#### *Clinical history*

In February 1946 a married woman, aged 48 years, with no significant previous or family history of illness, developed a cold which was associated with a productive cough, pink frothy sputum and pain in the left side of the chest. Following this episode her breathing remained asthmatical in type and attacks of increased difficulty in breathing associated with blood-streaked sputum occurred about once a fortnight. At this time she was treated at home by her doctor with the usual remedies for asthma, and with sulphonamides. In May 1946 she was admitted to a hospital near her home with an exacerbation of symptoms. Her condition was diagnosed as lobar pneumonia and she was treated with a sulphonamide drug. There was rapid clinical improvement and she was able to go home. Within a few weeks she was readmitted to the same hospital with a recurrence of symptoms. On this occasion there was no evidence of pulmonary consolidation, and after treatment with antispasmodics and penicillin inhalations she again returned home. In July 1946 a further relapse occurred and she was admitted to the London Hospital on 30th July. On admission she was very cyanosed, pale, clammy and collapsed, with a temperature of 99° and slight pitting oedema of the legs.

*Respiratory system.* Respirations 48; prolongation of expiration; crepitations with a few rhonchi heard in all areas.

*Cardiovascular system.* Marked venous congestion. Pulse 130, of poor volume. There was no other abnormality except that the blood pressure was not recordable on admission and there was no subsequent record.

#### *Clinical investigations*

*X-ray reports on chest.* 6.8.46. "There is an opacity at the left apex with a small cavity in the middle. The rest of the lung fields show faint mottling



and the individual opacities vary in size from about 1 to 3 mm. They are most numerous in the lower half of the chest." 18.8.46. "No material change in the appearance of the chest."

*Blood.* Hb. 81 per cent. Leucocytes 17,200 (neutrophils 36, eosinophils 41, lymphocytes 20, monocytes 3 per cent.). Erythrocyte sedimentation rate (corrected) 46 mm./1 hour.

*Sputum.* Moderate growth of hæmolytic streptococci on culture. No tubercle bacilli found.

*Urine.* Cloud of albumin on admission; none noted on three subsequent examinations.

#### *Clinical course*

As the asthma did not respond to adrenaline she was given "Cardophylin" (aminophylline) on several occasions, with some relief, and at times morphine, atropine and oxygen. The eosinophilia in the blood persisted throughout the illness. She improved slightly during the first few weeks in hospital and then gradually became weaker, dying five weeks after admission. She had slight diarrhœa without blood or mucus in the last week. Irregular fever, usually between 99 and 100°, persisted throughout the illness.

#### *Post-mortem examination (P.M. 268/46)*

*Summary.* Heart failure: myocarditis: disseminated pulmonary, myocardial, splenic and renal lesions.

*Lungs.* Numerous groups, up to 1 cm. in diameter, of confluent, brownish-yellow nodules throughout the lungs, with one round area of hæmorrhage, 2 cm. in diameter, like a circular infarct, near one of the groups, and a few irregularly shaped granular brown areas of consolidation, 2.5 cm. in diameter, in the left lower lobe. Œdema and congestion of the posterior parts of both lungs; obsolete tuberculosis in the left lower lobe, and a small cavity 1 cm. in diameter near the apex of the left upper lobe.

*Heart.* Recent infarct-like areas of yellow necrosis with hæmorrhagic borders involved the whole thickness of the right two-thirds of the posterior wall of the left ventricle and the sub-endocardial half of the remainder of the ventricle. All coronary arteries and their visible branches normal. Very numerous greyish-yellow submiliary flecks under the endocardium of the upper part of the right ventricle and sub-epicardial streaks in the apical region of both ventricles. Valves normal. Two small portions of ante-mortem thrombus in left ventricle.

Central congestion with paradoxical lobulation of liver. Two firm pink infarcts of spleen. Numerous thin grey vertical streaks and submiliary grey nodules throughout the cortex of both kidneys, especially beneath the capsule.

Weight of organs: heart 297 g., liver 1622 g., kidneys 291 g., spleen 170 g., pancreas 106 g., thyroid 9 g., thymus 7 g., ovaries 9 g., pituitary 0.8 g., brain 1100 g., body weight 36.5 kg.

DISSEMINATED FOCAL NECROSIS IN ASTHMA



FIG. 1.—Section from lower lobe of left lung, showing a central area of necrosis and peripheral zone of radiating spindle cells, multinucleated giant cells and lymphocytes. Hæmatoxylin and eosin.  $\times 80$ .

FIG. 2.—Part of lower edge of lesion shown in fig. 1.  $\times 230$ .

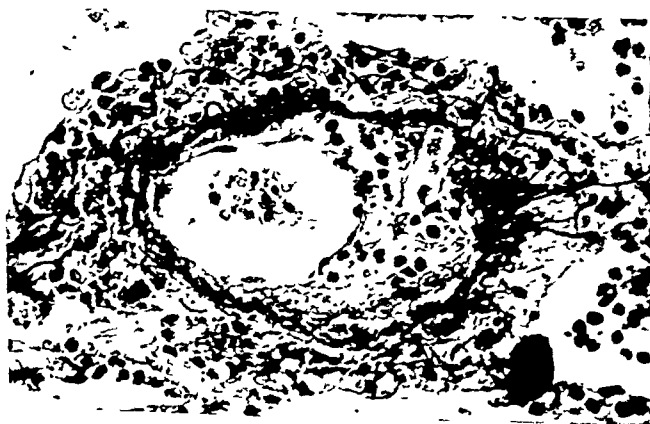
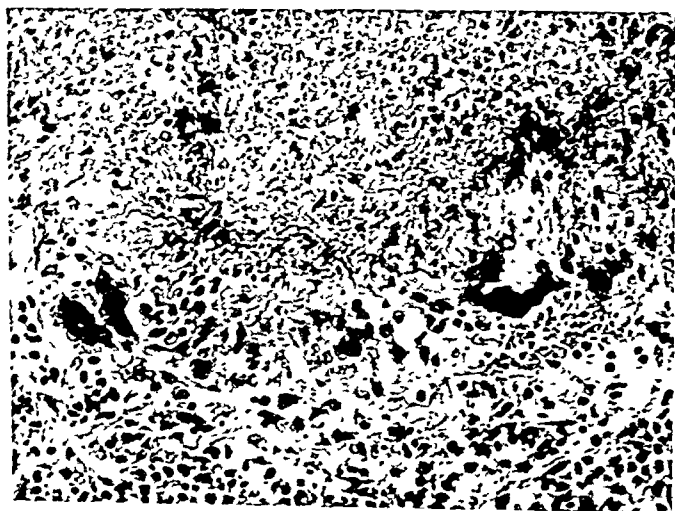


FIG. 3.—Small pulmonary arteriole showing endarteritis. Hart's elastic stain.  $\times 280$ .



DISSEMINATED FOCAL NECROSIS IN ASTHMA

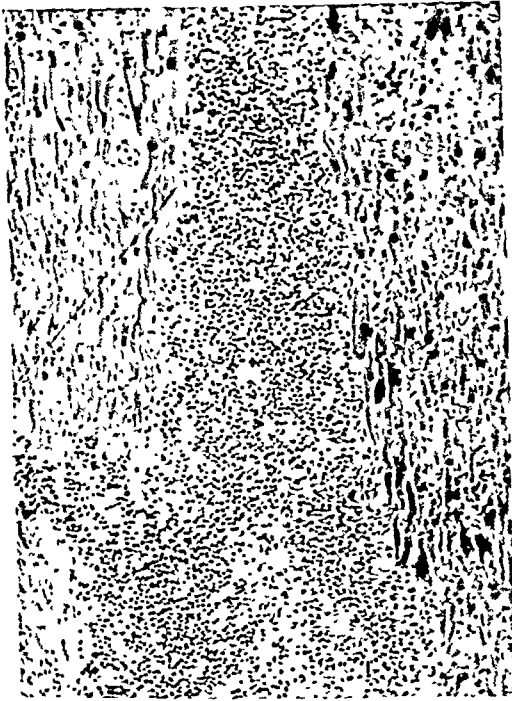


FIG. 4.—Extensive infiltration of myocardium (interventricular septum). Hæmatoxylin and eosin.  $\times 80$ .

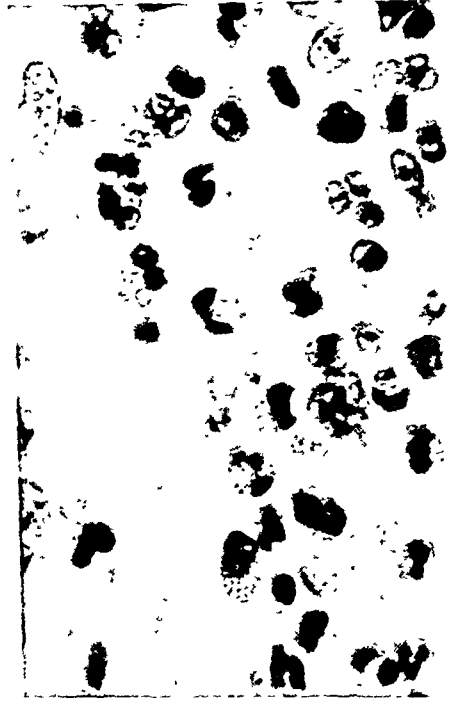


FIG. 5.—Infiltration of interventricular septum, showing eosinophil leucocytes.  $\times 1000$ .



FIG. 6.—Kidney. Infiltration of interstitial tissue with eosinophil leucocytes and hæmorrhage into Bowman's capsule of one glomerulus. Hæmatoxylin and eosin.  $\times 80$ .



*Microscopical examination*

Portions of lung (left lower lobe, two blocks ; right lower lobe and left upper lobe, 2 blocks), heart (left ventricle, two blocks ; interventricular septum and right ventricle, two blocks), spleen, liver and kidney were fixed in formol-saline, embedded in paraffin and stained with hæmatoxylin and eosin, Weigert's iron hæmatoxylin and van Gieson, Hart's elastic stain, Gram and Ziehl-Neelsen. A selection of slides was also stained with Mallory's phosphotungstic acid-hæmatoxylin.

The most remarkable features are the eosinophilic infiltration and vascular lesions in all the organs examined, and the focal lesions in the lungs. No bacteria can be demonstrated in any of the sections.

*Lungs.* Throughout both lungs there are irregular areas of necrosis, ranging in size from  $250\ \mu$  to  $1.2 \times 1\text{ cm.}$ , in which the alveolar spaces contain large numbers of necrotic eosinophil polymorphonuclear leucocytes and some large mononuclear cells ; many also contain tangled masses of fibrin filaments. The periphery of these areas is densely infiltrated with eosinophils. In some of the smaller necrotic foci ( $400\ \mu$  diameter) the eosinophilic infiltration is associated with radiating spindle cells, multinucleated giant cells with either central or peripheral nuclei, lymphocytes and scanty plasma cells (figs. 1 and 2). Such focal necrotic granulomatous areas are perhaps the most significant of the lesions present. In a few areas proliferation of fibroblasts in inter-alveolar septa, occasionally extending into alveolar spaces, is also seen. Lesions of polyarteritis nodosa type are present in sections from the left lower and right lower lobes. Some of these are in the acute stage, with eosinophilic infiltration and focal necrosis of the walls, with or without recent thrombi in the small and medium-sized arteries. Fibrin deposition in the vessel walls is absent. Many vessels show partial obliteration of the lumen by granulation tissue (fig. 3), and in some, this is associated with fibrosis of the wall, indicating a late stage of polyarteritis. In sections from the right lower lobe a typical area of hæmorrhagic infarction associated with recent thrombosis in a medium-sized artery is present. However, the frequency of the characteristic areas of necrosis in comparison with vascular thrombosis suggests that some at least are not infarcts. The submucosa of many bronchi and bronchioles is heavily infiltrated with eosinophil leucocytes and with fewer plasma cells and lymphocytes.

*Heart.* In all areas examined the interstitial tissue of the necrotic areas is unevenly stuffed with degenerating and dead eosinophil leucocytes mixed with smaller numbers of round cells of other kinds. A similar inflammatory cellular exudate extends into the adjacent living muscle and infiltrates the pericardium and endocardium. In all areas the eosinophil leucocyte predominates (figs. 4 and 5). Stages of panarteritis as described in the lungs are present here too, but in some of the smaller vessels the whole thickness of the wall is impregnated with fibrin. Multinucleated giant cells of the rheumatic

lungs) revealed similar necrotising vascular lesions, diffuse eosinophilic infiltration and focal granulomatous areas. They equate the condition with Loeffler's syndrome. Harkavy (1943) has described a special group of cases of bronchial asthma characterised clinically by a prolonged course, eosinophilia and involvement of other organs besides the lungs, in particular the serous cavities. At necropsy four of these cases displayed arterial lesions as well as acute and chronic inflammatory changes in the affected organs. Fugitive pulmonary infiltrations had been present during life and were attributed to vascular changes or to infiltration of the inter-alveolar septa with eosinophils, polymorphonuclears and lymphocytes. No focal granulomatous lesions were described by him. He postulates that a hypersensitivity which in the usual case of asthma is restricted to the bronchial mucosa becomes widespread in these fatal cases, affecting vessels in many organs.

It is tempting to assume that cases such as the one here described and that described by Bayley *et al.* are a link between the benign condition described by Loeffler and the usually fatal polyarteritis nodosa. Such an assumption, however, merely indicates that the manifestations of anaphylaxis may be widespread or localised. It does not support a thesis that these conditions are all manifestations of one disease.

#### SUMMARY

A case is recorded which clinically resembled a protracted Loeffler's syndrome and was eventually fatal. At necropsy disseminated lesions of an unusual type were found, some being of the polyarteritis nodosa type but with additional widespread eosinophilic infiltration and necrotic granulomatous areas in the lungs. The significance of the case is briefly discussed.

I am indebted to Professor D. S. Russell for much help and encouragement in the preparation of this paper and to Dr Horace Evans for granting me access to the clinical notes.

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576.8.097.2:576.851.57 (*Cl. welchii*)

## THE KAPPA AND LAMBDA ANTIGENS OF *CLOSTRIDIUM WELCHII*

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AFTER Macfarlane and MacLennan (1945) demonstrated the existence in *Clostridium welchii* type-A filtrates of a substance, probably a collagenase, capable of softening muscle by destroying its collagen and reticulin scaffolding (see also Robb-Smith, 1945), Oakley, Warrack and van Heyningen (1946) showed that this substance, which they called kappa ( $\kappa$ ) toxin, was antigenically distinct from the  $\alpha$  and  $\theta$  toxins and the hyaluronidase produced by *Cl. welchii* type A, and that  $\kappa$  toxin and its antitoxin could be titrated with fresh guinea-pig muscle, collagen "paper" and "azocoll" as indicators. The present communication deals with some other indicators for  $\kappa$  toxin, with its distribution among the *Cl. welchii* types, and with another antigen ( $\lambda$ ) produced by type B and type "Bosworth", which is hereafter called type E.

### INDICATORS FOR KAPPA TOXIN

#### 1. Swelling on intramuscular injection

When  $\kappa$ -containing *Cl. welchii* filtrates in which  $\alpha$  and  $\theta$  toxins have been neutralised by sera without  $\kappa$  antitoxin are injected intramuscularly into guinea-pigs or mice, a tense local swelling appears in a few hours and persists for about two days. Similar swellings were noted by Fredette and Frappier (1946) after intramuscular injection of "non-toxic" *Cl. welchii* type-A filtrates into guinea-pigs. *Cl. welchii* antisera will inhibit the production of these swellings and sera can therefore be calibrated against a standard for their swelling-inhibiting power, with slight swelling of the injected limb as the standard indicating effect.

*Method.* *Cl. welchii* type-A filtrates are concentrated by saturation with ammonium sulphate, followed by dialysis of the precipitate against running tap water and the addition of sodium chloride to 1 per cent.;  $\alpha$  and  $\theta$  toxins are neutralised by the addition of sera without detectable  $\kappa$  antitoxin. Serum R 8531 was given an arbitrary value of 180 units, and the test dose of filtrate for muscle-swelling tests determined at 30 units by a method similar to that used for testing sera. Sera are then calibrated as follows. The test dose at 30 units is placed in deposit glasses, varying quantities of the test sera are added and the volume made up with 1 per cent. saline to 2 c.c. After mixing,



the mixtures are allowed to stand for half-an-hour and 0.4 c.c. is injected into the left thigh muscles of large ( $>20$  g.) mice. Twenty-four and 48 hours after injection the mice are examined in the following way. An assistant holds the mouse by the skin of the back of the neck and proffers the tail to the reader, who takes the middle of its length between the thumb and second and third fingers of his left hand. He then steadies his right hand by holding the tip of the tail between the thumb and second finger of his right hand, and palpates the left and right thighs alternately between the pulps of his index fingers, thus using the uninjected thigh as a control. The degree of swelling is estimated as none (-), doubtful ( $\pm$ ), slight ( $\pm$ ), or marked (+), and the end-point mixture is determined as the one producing  $\pm$  or + in half the mice injected. Non-specific reactions, often marked at 24 hours, have usually disappeared by 48 hours, when the final reading is taken.

Table I shows that antisera of *Cl. welchii* types A, C, D and E neutralise the muscle-swelling factor in type-A filtrates in proportion to their  $\kappa$ -antitoxin content; it is therefore probable that  $\kappa$  toxin is responsible for muscle swelling.

TABLE I

Comparison of anti- $\kappa$  and anti-swelling values of *Cl. welchii* types A, C, D and E antisera to show that  $\kappa$  toxin causes local swelling on intramuscular injection

Serum	Serum values	
	Anti- $\kappa$	Anti-swelling
R 8537	180	180
A 16836	100	110
R 3952	250	260
EX 770	350	420
H 3044	550	420
H 4415	2000	1650

Histological examination shows that the swelling is due to low-grade inflammation and œdema of the connective tissue, with destruction of reticulin and collagen.

## 2. Lesions on intracutaneous injection

Intracutaneous injection into guinea-pigs of  $\kappa$ -containing *Cl. welchii* type-A filtrates free from  $\alpha$  and  $\theta$  toxins leads in about 48 hours to the development of an ill-defined necrotic and hæmorrhagic lesion. Histological examination shows that collagen in the injection area no longer stains red with van Gieson's stain, that elastin fibres can readily be demonstrated passing through the otherwise unstained area, and that extensive hæmorrhages are common.

Serum values for inhibition of the necrotising effect can be determined by making up filtrate-serum mixtures in the usual way, allowing them to stand for half-an-hour, and injecting 0.2 c.c. intracutaneously. After two days the guinea-pig is killed, the skin stripped

off, and the extent of the lesion read from the deep surface of the skin, taking a lesion about  $2 \times 2$  mm. as the standard indicating effect.

Attempts to use the necrotising effect as an indicator in serum-value tests were not very successful, because the accuracy of the test is very low even if high concentrations of filtrate are used. In view of the nature of the lesion it seems likely that it is due to  $\kappa$  toxin; the serum-value tests support this view, but are not sufficiently reproducible to prove it.

### 3. Death on intravenous injection

When highly concentrated  $\kappa$ -containing filtrates, free from  $\alpha$  and  $\theta$  toxin, are injected intravenously into mice, death follows within 24 hours, with free bleeding from the nose as the only important symptom. Post-mortem examination shows extensive hæmorrhages in the lungs; histologically the reticulum in the lung is severely damaged and presumably the unsupported capillaries leak blood into the alveoli.

Attempts to use death or the production of small hæmorrhages in the lungs after intravenous injection as indicators for serum-value tests gave answers suggesting that  $\kappa$  is the responsible toxin, but the reproducibility of the results is not high enough for proof.

### 4. Attack on gelatin

In view of the close relationship between collagen and gelatin, it would be expected that collagenases would attack gelatin. After several attempts to use 25 per cent. gelatin pellets as indicators, we devised a gelatin-agar column method. This was a modification, suitable for serum-value determinations, of the method for detecting gelatinase production by bacterial colonies (Frazier, 1926).

The method depends on the fact that if 15 per cent. mercuric chloride in 20 per cent. hydrochloric acid is poured on the top of a 1 per cent. agar column containing 0.4 per cent. gelatin, a white precipitate is formed which spreads down the column as the mercuric chloride diffuses through it. Two precipitates are in fact formed, one extending down the tube faster than the other; the faster is presumably due to the hydrochloric acid and the slower to the mercuric chloride. If the gelatin-agar column is previously treated with a gelatinase the gelatin is attacked and rendered unprecipitable by acid mercuric chloride; the amount of gelatinase in the filtrate used can be estimated from the depth of the clear zone at the top of the column after mercuric chloride treatment.

The attack on gelatin by  $\kappa$ -containing filtrates is inhibited by *Cl. welchii* antisera, and these sera can be calibrated against a standard for anti-gelatinase activity by the following method (fig.). Make filtrate-serum mixtures in the usual way, allow them to stand for

half-an-hour, and pour them on the top of gelatin-agar columns; incubate at 37° C. in a water-bath overnight. Next morning pour off the filtrate-serum mixtures and replace them with acid mercuric chloride; read after two hours. Take a small clear zone at the

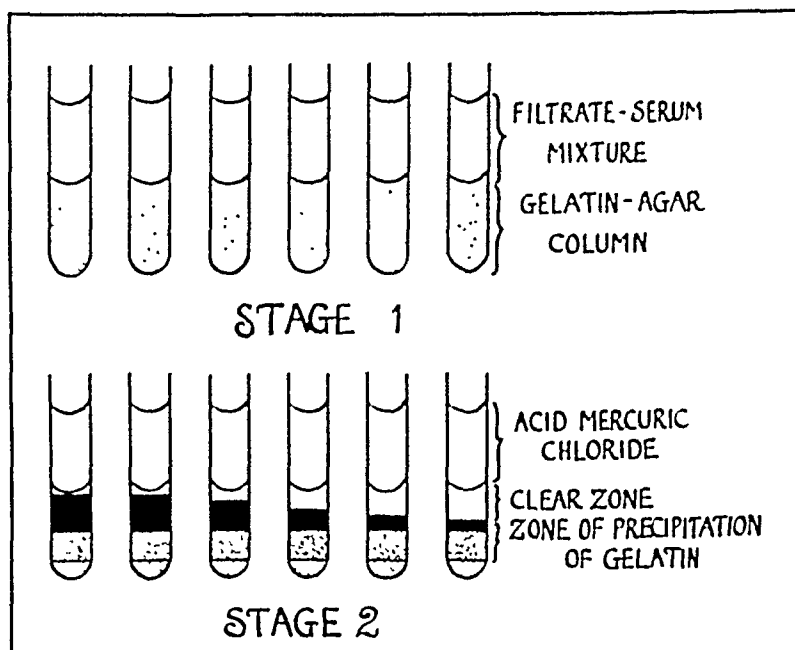


FIG.—Anti-gelatinase testing by the gelatin-agar method.

surface as the standard indicating effect. A positive blank of small dimensions is always obtained; this may be due to leaching out of the gelatin by water, or perhaps to the presence in the filtrates of another gelatinase to which our sera have little antibody.

If the standard serum R 8537 is given a value of 180 units, values of other sera determined against  $\kappa$ -containing filtrates lie sufficiently close to their anti- $\kappa$  values to make it highly probable that  $\kappa$  toxin attacks gelatin (table II).

TABLE II

*Comparison of anti- $\kappa$  and anti-gelatinase values of sera to show that  $\kappa$  toxin attacks gelatin*

Serum	Serum values	
	Anti- $\kappa$	Anti-gelatinase
R 8537	180	180
LX 251	650	650
R 7843	360	350
EX 770	360	360
R 7480	280	240
RR 3518	480	400

# DISTRIBUTION OF SUBSTANCES ATTACKING AZOCOLL AMONG THE CL. WELCHII TYPES

Throughout this paper the customary differentiation of *Cl. welchii* into types A, B, C, D, based on toxigenicity, will be followed; Bosworth's strains (1940-43) will be called type E.

It can readily be shown that all types of *Cl. welchii* produce substances attacking azocoll. Type-A strains, some type-C and type-D strains and all type-E strains soften muscle and disintegrate collagen paper. Filtrates from type-B strains consistently fail to attack collagen paper or to soften muscle.

Serological examination of filtrates from type-A and type-E strains with collagen paper as the indicator show that the substance effective in this test is  $\kappa$  toxin; similar results are obtained in azocoll tests on type-C and D filtrates that are too weak to use in muscle or collagen-paper tests (table III).

TABLE III

*Serum values against filtrates from three different types of Cl. welchii to show that all produce  $\kappa$  toxin (muscle tests)*

Serum	Serum values against filtrates		
	Type A	Type C	Type D
R 8537	180	180	180
LX 251	750	800	800
H 3044	550	500	500
H 2917	850	800	800
R 7843	360	410	400
R 6981	30	44	30

Serum values against type-B filtrates with azocoll as indicator give results showing no relationship to the anti- $\kappa$  values of the sera used; similar results are obtained with some type-D filtrates; the serum values against type-B filtrates and such type-D filtrates agree very well, and are presumably determined against the same antigen in both cases. Clearly type-B strains and some type-D strains produce a substance that will attack azocoll and hide powder, but not muscle or collagen paper (table IV).

TABLE IV

*Comparison of anti- $\kappa$  values and of serum values against type-B filtrates, in azocoll tests, to show existence of  $\lambda$*

Serum	Anti- $\kappa$ values	Values against type-B filtrates
R 8537 . . . .	180	30
R 7480 . . . .	280	5000
RR 3518 . . . .	480	900
Normal horse (fresh) . . . .	<1	1500
Normal horse (old) . . . .	<1	<4
Normal sheep (fresh) . . . .	<1	1000

It will be noted that fresh normal sera neutralise the azocoll-attacking substance present in type-B filtrates; this, and the absence from some  $\kappa$  antisera of antibody against the azocoll-attacking substance in type-B filtrates, makes it possible to show that type E produces both  $\kappa$  and the new substance, denoted by  $\lambda$  ( $\lambda$ ). For if sera are tested against type-E filtrates in azocoll tests after neutralisation of  $\lambda$  with the normal serum, the answers are equal to the anti- $\kappa$  values. If the  $\kappa$  toxin is neutralised with sera containing no anti- $\lambda$ , the values determined run parallel with the anti- $\lambda$  values (table V).

TABLE V

*Comparison of anti- $\kappa$  and anti- $\lambda$  values of sera with those obtained against type-E filtrates in azocoll tests to show that type E (Bosworth) produces both  $\kappa$  and  $\lambda$*

Serum	Serum values			
	Anti- $\kappa$	Anti-E ( $\lambda$ -neutralised)	Anti- $\lambda$	Anti-E ( $\kappa$ -neutralised)
R 8537	180	180	30	30
R 7480	280	260	5000	4200
RR 3518	480	520	900	1000
R 1974	380	370	<4	<4
R 7532	450	450	4000	4500
EX 770	350	300	32	30

These experiments show clearly that hide powder and its derivative azocoll are not satisfactory indicators for collagenases. In some way during their preparation they have been altered so that they are attacked by enzymes— $\lambda$ , trypsin, chymotrypsin (Todd, 1947)—that normally attack collagen with extreme difficulty. It is true that if a preparation fails to attack azocoll it is very unlikely to attack collagen; so that negative results with azocoll as indicator are of value. Positive results are not necessarily due to the action of collagenases, and should not be regarded as evidence of their presence without independent confirmatory evidence. The most specific indicator so far is collagen paper, but unfortunately it is relatively insensitive.

In their production of  $\kappa$  and  $\lambda$ , types A, B, C and E showed remarkable consistency; 11 type-A strains and 6 type-C strains all produced  $\kappa$ ; of 50 type-B strains, 49 produced  $\lambda$ , and the remaining type-B strain (LD/1933) is degraded in other respects as it has ceased to produce  $\epsilon$ . All 8 type-E strains produced both  $\kappa$  and  $\lambda$ . By comparison type-D strains appeared very heterogeneous. Thus 6 produced no substances attacking azocoll, 8 produced  $\kappa$ , 6 produced  $\lambda$ , and 15 produced perhaps both  $\kappa$  and  $\lambda$ . This naturally suggests that the type-D strains that produce  $\lambda$  may be derived from type-B strains by loss of  $\beta$  toxin; so far we have not obtained any conclusive evidence that this is so.

*Action of  $\lambda$  on gelatin.* Though gelatin-agar tests for serum values against  $\lambda$ -containing filtrates are not very satisfactory, as the end-points are very difficult to determine, they give general support for the view that  $\lambda$  attacks gelatin.

*Effect of medium and time of harvesting on  $\kappa$  and  $\lambda$  production*

Both the medium and the time of harvesting markedly affect the proportions of  $\kappa$  and  $\lambda$  antigens produced by strains of *Cl. welchii*. Thus in ordinary meat broths production of  $\lambda$  by *Cl. welchii* type B is maximal at about 5 hours and decreases thereafter. In such meat broths types A and C and some strains of type D produce  $\kappa$  only; type B produces  $\lambda$ , type E,  $\kappa$  and  $\lambda$ . In the medium devised by Adams and Hendee (1945) for production of *Cl. welchii*  $\alpha$  toxin, types A and C and some strains of type D still produce  $\kappa$ , but in much reduced amount; production of  $\kappa$  by type-E strains is completely inhibited. On the other hand production of  $\lambda$  by types B and E and some strains of type D is greatly increased.

*Antigenicity of  $\lambda$*

The observation that  $\lambda$  is "neutralised" by fresh normal sera (horse, sheep, cow and rabbit) raises the question of its antigenicity. Refining of normal sera by Pope's process (1939) completely destroys all  $\lambda$  inhibitor; sera from horses immunised with type-B filtrates retain much activity after similar treatment. Electrophoretic examination of normal sera (table VI) showed that all the  $\lambda$ -neutralising

TABLE VI

$\lambda$  "antibody" in electrophoretic fractions of sera to show antigenicity of  $\lambda$

Material examined	$\lambda$ "antibody" in electrophoretic fraction			
	A	A + $\alpha$	$\beta + \gamma$	$\gamma$
Normal serum before pepsinisation .	10	80	<3	<3
" " after pepsinisation .	<3	<8	<3	<3
Immune serum before pepsinisation .	<4	125	60	35
" " after pepsinisation .	<4	<4	57	21

material was in the albumin plus  $\alpha$ -globulin fraction, and was completely destroyed by pepsin treatment at pH 4. Similar examination of immune sera showed that the  $\lambda$ -inhibiting substances appeared in three fractions, albumin plus  $\alpha$ ,  $\beta$  plus  $\gamma$ , and  $\gamma$ . The part in the albumin plus  $\alpha$  fraction was like that in normal serum destroyed by pepsin treatment at pH 4; those in the  $\beta$  plus  $\gamma$  and  $\gamma$  fractions resisted this treatment and therefore behaved like true antitoxins. Moreover crude sera from normal and immunised horses inhibit not only the action of  $\lambda$  on azocoll, but also similar action by filtrates from *Clostridium bifermentans* and *Clostridium sordellii*. When these

sera are refined by Pope's process (table VII), normal sera lose both  $\lambda$  inhibitor and the substance or substances neutralising *Cl. bifermentans* and *Cl. sordellii* filtrates; sera from horses immunised with  $\lambda$ -containing filtrates lose their capacity to neutralise filtrates of *Cl. bifermentans* and *Cl. sordellii* in azocoll tests, but retain part of their capacity to

TABLE VII

To show that  $\lambda$  antibody is specific and does not neutralise the substances in *Cl. bifermentans* and *Cl. sordellii* filtrates that attack azocoll and are inhibited by normal serum

Serum	$\lambda$ inhibitor in		<i>Bifermentans</i> inhibitor in		<i>Sordellii</i> inhibitor in	
	fresh serum	refined serum	fresh serum	refined serum	fresh serum	refined serum
RR 5863	730	260	100	<4	100	<4
RR 5559	5000	3000	100	<4	200	<4
RR 6380	1350	750	200	10	200	20
RR 5494	780	8	50	<4	100	<4

neutralise  $\lambda$ , which is therefore much more specific than that of the inhibiting substances in normal serum. Since injection of  $\lambda$ -containing filtrates into horses leads to the development in the serum of neutralising substances not present in normal sera, and since these substances react to pepsin treatment at pH 4 exactly as antitoxins react, there can be no doubt that  $\lambda$  is antigenic.

## SUMMARY

*Cl. welchii*  $\kappa$  toxin attacks gelatin. It produces local swelling on intramuscular injection and a local hæmorrhagic and necrotic reaction on intracutaneous injection. It is probably lethal on intravenous injection. Death after intravenous injection is associated with massive hæmorrhages into the lungs.

Toxin production by different types of *Cl. welchii* under suitable conditions may be summarised thus :—

Types A and C produce  $\kappa$  toxin.

Type B produces an antigen lambda ( $\lambda$ ), not previously described, which attacks hide powder and azocoll but fails to attack collagen; it probably attacks gelatin. Fresh normal sera contain a  $\lambda$  inhibitor distinct from  $\lambda$  antibody produced by immunisation.

Type E produces both  $\kappa$  and  $\lambda$ .

Type-D strains show great diversity. Some produce  $\kappa$  and some  $\lambda$ , some possibly both and some neither.

It must be emphasised that hide powder and azocoll are not reliable as indicators for collagenases, because they are attacked by enzymes that do not attack collagen.

We should like to thank Mr A. Thomson and Miss H. E. Ross for many bacterial filtrates, Mr A. J. Harms for much assistance in refining sera, Mr P. A. Charlwood for carrying out the electrophoretic separations, and Miss E. Hart for considerable technical assistance.

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## SHORT ARTICLES

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### A CASE OF CEREBRAL CYSTICERCOSIS

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(PLATE XC)

Although cerebral cysticercosis has been the subject of a number of papers since MacArthur (1933-34) first drew attention to this condition as a cause of epilepsy, few cases have been reported in detail amongst Indian civilians (Minchin, 1937; Menon and Veliath, 1939-40). The following instance presents some features which are worthy of record.

The patient, a Hindu girl aged 14 years, had been adopted by a village family four years before she became ill. This family did not keep pigs nor ate pork, but the possibility of infection at some period prior to the child's adoption could not be ruled out. When brought to a mission hospital near her home three weeks before her death, she gave a history of fever and severe headache for four months, with steady deterioration of her eyesight until she could no longer distinguish between day and night. On examination, the pupils were widely dilated and did not react to light, and papilloedema was noted. She was sent to Vellore for further ophthalmic investigation, when the presence of well marked papilloedema in both optic discs with exudate scattered over the retinae was confirmed. The Kahn test was negative. Her physical condition rapidly deteriorated, making a detailed neurological investigation impossible, and she died six days later.

Autopsy was performed 36 hours after death, the body having been preserved in a refrigerator. The subject was a well nourished young girl, 4 ft. 6 in. in height, with slight generalised oedema, most pronounced on the face. The lungs showed hypostasis and early bronchopneumonia. The spleen was considerably enlarged (350 g.), adherent to the stomach, red, soft and friable, with much dark pigment but no malarial parasites microscopically. The small intestine contained a *Taenia solium* 55 cm. in length. Careful search failed to reveal the presence of cysts in any of the muscles, the eyes or the thoracic or abdominal organs. The only other pathological changes found were in the brain. The meninges were free from adhesions. On the surface of the brain were numerous small nodules 1.5 mm. in diameter and others could be felt as small firm lumps within its substance. A small grape-like cystic mass was adherent to the arachnoid immediately to the right of the middle line over the parietal area. A number of tiny cysts were found in the grey matter (fig. 1), one being present in the thalamus and 7 in the cerebellum. In all, 213 cysts were counted, 121 on the right side, 92 on the left. They could be easily shelled out. Each possessed a thin semi-translucent membranous wall and contained clear fluid and a single scolex (fig. 2). The scolex had a crown of 24 radiating hooklets (fig. 3) and 4 suckers. Its short hooklets averaged  $109\ \mu$  in length, its long hooklets  $146\ \mu$ . These findings fall within the range given by Maplestone (McRobert, 1944) for the cysticercus of *Taenia solium*.

Microscopic examination of a portion of the brain containing a cyst shows three zones.

1. In the centre, the outline of the parasite can be seen, with its hooklets and suckers surrounded by coagulated albuminous material. There is no evidence of calcification of the parasite.

2. Surrounding this there are masses of polymorphonuclear leucocytes entangled in fibrin with a few macrophages. Just outside this layer in some cysts there are patchy collections of epithelioid cells and occasional foreign-body giant cells. The extent and degree of inflammatory reaction vary in different cysts.

3. The outermost zone consists of a layer of granulation tissue where there are actively proliferating fibroblasts, many young capillaries and scattered clumps of lymphocytes and plasma cells, with occasional polymorphonuclear leucocytes. This zone, the thickest, merges with the surrounding brain tissue. The macroscopically normal brain lying between the cysts contains scattered collections of lymphocytes.

An X-ray of a thin slice of the brain shows the negative shadows of the cysts so that it is doubtful if these would have shown through the skull before death. There is no radiological evidence of calcification in any of the cysts.

### Summary

A rapidly fatal case of cerebral cysticercosis is reported. The patient, a Hindu girl aged 14, had symptoms suggestive of cerebral tumour. The adult worm was found in the small intestine. The cysts showed no evidence of calcification and the cysticerci were alive. An intense inflammatory reaction had developed around the cysts and was considered to be responsible for the symptoms.

We are indebted to Dr Thangavelu for the photomicrographs, to Mr Simon for preparing the histological sections and to Dr H. Lazarus for permission to report the case.

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616 . 34—008 . 87 : 595 . 722 (*Blatella americana*)

### A STUDY OF THE INTESTINAL FLORA OF *BLATELLA AMERICANA*

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The American cockroach, *Blatella americana*, is a common insect in this country, where warmth, darkness and organic detritus provide favourable conditions for its existence. It is a nocturnal insect and normally shuns the light, so that the few strays that may be seen in daylight give no indication of the density of its population in a given locality. It is often a pest in bakehouses, especially if coke is used for heating the ovens. Since it feeds voraciously on all kinds of dead organic matter and contaminates human foodstuffs with an evil-smelling slime, it may be at times a serious economic pest. Although we know of no records of cockroaches attacking human beings in these islands,

CEREBRAL CYSTICERCOSIS



FIG. 1.—Vertical sections of the brain to show the cysts mainly in the grey matter.  $\times c. \frac{1}{2}$ .



FIG. 2.—The scolex removed from one of the cysts.  $\times c. 100$ .

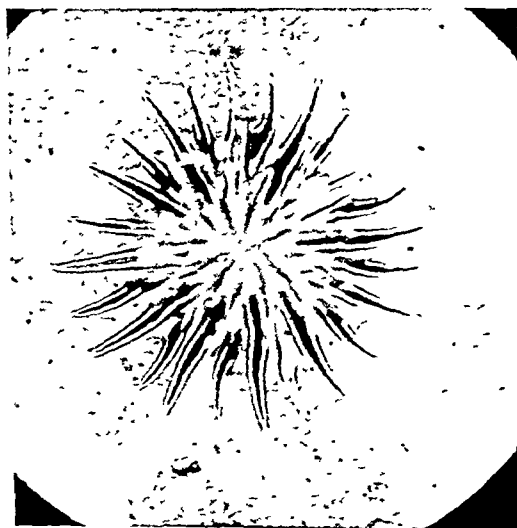


FIG. 3.—Crown of hooklets on the scolex, showing their arrangement.  $\times c. 250$ .



Moiser (1945) states that in Southern Rhodesia two species, *Blattella germanica* and *Oxyhaloa murrayi*, attack the natives at night, inflicting savage bites which leave "on the African's dark skin a white, almost circular scar, often much larger than a pin's head".

Steinhaus (1946) has summarised the findings of numerous workers about the occurrence of intracellular, bacterium-like symbionts in the fat-body of *B. americana* and mentions the finding of spirochaetes in its intestinal canal by Dobell and others. Moiser, who was working in a leper colony, reports the common finding of acid-fast bacilli, which he asserts are *Mycobacterium lepræ*, in the intestines of the local species, and suggests that those roaches may play an essential part in the transmission of leprosy. His work, however, has not been confirmed.

This study is concerned particularly with the normal bacterial flora of the insect's alimentary tract, and no search was made either for the symbionts in the fat-body, or for intestinal spirochaetes. Since the insect may contaminate human food with its excrement, a study of its intestinal bacterial flora for possible human pathogens seemed worth undertaking.

### Methods

Adult cockroaches were collected from the service duct underneath the Birmingham Medical School, and from various bakehouses, shops and kitchens in the suburbs of Harborne, Ladywood and Edgbaston. Specimens were killed by immersion for one minute in water at 80° C., and the alimentary tract was then immediately dissected out in its entirety. Cultures were sown on a variety of solid and liquid media from the contents of the crop, the mesenteron, the small intestine—including the proctodeum—and the large intestine. Some of these were incubated under aerobic, some under anaerobic, conditions at 20° C. Film preparations were made from each of the parts of the tract mentioned, and were stained by Gram's, Neisser's, and Ziehl-Neelsen's methods. All the media used had an initial pH of 7.5, and the cultures were incubated for 3 or 4 days before plating out the liquid cultures on various agar media.

Microscopic examination of the film preparations from all parts of the tract revealed a flora predominantly of Gram-negative bacilli. No acid-fast bacteria were seen in any of the many preparations examined.

From the large number of primary isolations obtained, 19 cultures of Gram-negative bacilli were finally selected, on the basis of their growth and colony characters and their fermentative action on glucose, lactose, mannitol and sucrose, for a detailed study of their biochemical activities. These bacilli were found to belong to four chief groups, three of which contained two or more sub-groups.

**Group 1.** Bacilli having no fermentative action upon any of the carbohydrates tested.

TABLE I  
*Biochemical reactions of Gram-negative bacilli, group 1*  
(sub-groups 1 and 2)

Sub-group	Motility	Indol	Methyl red	Voges-Proskauer	Lead acetate	Nitrate reduction	Gelatin liquefaction	Litmus milk
1	—	—	—	—	—	—	—	Alk.
2	+	—	—	—	+	÷	÷	Dig.

**Group 2.** Bacilli fermenting *lævulose* only, with production of acid but no gas. Motile. Indol, methyl red, and Voges-Proskauer negative. No blackening of lead acetate. Nitrates reduced. No liquefaction of gelatin. No change in litmus milk.

**Group 3.** Bacilli attacking various carbohydrates with production of acid but no gas.

TABLE II

*Biochemical reactions of Gram-negative bacilli, group 3  
(sub-groups 1-5)*

Sub-group	Xylose	Glucose	Levulose	Galactose	Lactose	Maltose	Sucrose	Raffinose	Mannitol	Dulcitol	Sorbitol	Inulin	Salicin	Asparagin	Dextrin	Glycerin	Starch	Motility	Indol	Methyl red	Voges-Proskauer	Lead acetate	Nitrate reduction	Gelatin liquefaction	Litmus milk
1	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	+	+	+	+	+	+	+	Alk.
2	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	+	+	+	+	+	+	+	—
3	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	+	+	+	+	+	+	+	A
4	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	+	+	+	+	+	+	+	Dig.
5	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	+	+	+	+	+	+	+	—

Each of the strains in sub-group 5 (table II) produced a bright red, diffusible pigment in liquid cultures, which interfered with the reading of the tests and made a correct interpretation of three of them (marked ?) impossible. We cannot be sure of the validity of all the sugar fermentation results, but each culture showed a definite acid change when tested with litmus paper.

**Group 4.** Bacilli attacking various carbohydrates with the production of acid and gas.

TABLE III

*Biochemical reactions of Gram-negative bacilli, group 4  
(sub-groups 1-4)*

Sub-group	Xylose	Glucose	Levulose	Galactose	Lactose	Maltose	Sucrose	Raffinose	Mannitol	Dulcitol	Sorbitol	Inulin	Salicin	Asparagin	Dextrin	Glycerin	Starch	Motility	Indol	Methyl red	Voges-Proskauer	Lead acetate	Nitrate reduction	Gelatin liquefaction	Litmus milk *
1	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	—	AG	—	—	AG	AG	—	+	+	+	+	+	+	A
2	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	—	AG	—	—	AG	AG	—	+	+	+	+	+	+	BC
3	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	—	AG	—	—	AG	AG	—	+	+	+	+	+	+	A
4	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	—	AG	—	—	AG	AG	—	+	+	+	+	+	+	AC

\* BC Indicates that milk was bleached and clotted; AC indicates acid and clot.

All the cultures for the sugar fermentation reactions were sown into 1 per cent. sugar-peptone-water media and incubated at 20° C. for 21 days before the final readings were made.

The primary anaerobic cultures were plated on Lemco agar after 4 days' incubation and the plates then incubated at 20° C. in the anaerobic jar. Eight cultures were finally selected for further study on the basis of their growth and colony characters. These were all motile, Gram-positive bacilli, which formed subterminal oval spores and produced slight blackening in cooked-meat broth. No other types of Clostridia were encountered. Two of these eight strains gave identical biochemical reactions; the reactions of the seven strains are set out in table IV.

From this table it would appear that these Clostridia may be separated into three ill-defined groups.

In addition to the bacilli described, strains of *Bacillus subtilis* and *Bacillus megatherium* were cultivated from each of the roaches examined.

TABLE IV

Biochemical reactions of seven strains of *Clostridia*

Gelatin liquefaction	Digestion of serum	Litmus milk	Glucose	Lactose	Maltose	Sucrose	Mannitol	Salicin
—	—	—	AG	—	—	—	—	—
—	+	—	AG	—	—	—	—	—
+	+	—	AG	—	—	—	—	—
—	—	—	AG	—	AG	—	—	—
—	—	Digestion	AG	—	AG	—	—	—
—	—	Digestion	—	—	—	—	—	—
—	+	—	—	—	—	—	—	—

Cocci were present only in small numbers, but a white, non-hæmolytic Gram-positive micrococcus which did not produce coagulase was cultivated from every insect, and two strains of streptococci were constantly recovered. Neither of these produced hæmolysin. One corresponded to *Streptococcus non-hæmolyticus* II of Holman's classification; the other to *Streptococcus faecalis*.

### Discussion

Apart from the fact that the bacilli in sub-group 1 of group 1 appear to correspond to Nyberg's (1934-35) *Bacterium alkaligenes*, none of the bacilli isolated have been found to correspond exactly with any of the recorded groups of Gram-negative bacilli. Presumably, therefore, they may be regarded, at any rate for the present, as non-pathogenic for man. A striking feature of the aerobic bacillary flora of the alimentary tract of *Blatella americana* is the high proportion of nitrate reducers.

### Summary

A study of the flora of the alimentary tract of the American cockroach has not demonstrated any bacteria belonging to any of the recognised groups of human pathogens. The flora shows a predominance of Gram-negative bacilli, which have been arranged in four groups on the basis of their biochemical activities.

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**Group 3.** Bacilli attacking various carbohydrates with production of acid but no gas.

TABLE II

*Biochemical reactions of Gram-negative bacilli, group 3*  
(sub-groups 1-5)

Sub-group	Xylose	Glucose	Levulose	Galactose	Lactose	Maltose	Sucrose	Raffinose	Mannitol	Dulcitol	Sorbitol	Inulin	Salicin	Asparagin	Dextrin	Glycerin	Starch	Motility	Indol	Methyl red	Voges-Proskauer	Lead acetate	Nitrate reduction	Gelatin liquefaction	Litmus milk
1	A	A	-	A	-	-	-	-	A	-	-	-	-	-	-	-	-	+	-	-	-	+	+	+	Alk.
2	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	+	+	+	+	+	+	+	-
3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	-
4	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	+	+	+	+	+	+	+	-
5	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	+	+	+	+	+	+	+	Dig.

Each of the strains in sub-group 5 (table II) produced a bright red, diffusible pigment in liquid cultures, which interfered with the reading of the tests and made a correct interpretation of three of them (marked ?) impossible. We cannot be sure of the validity of all the sugar fermentation results, but each culture showed a definite acid change when tested with litmus paper.

**Group 4.** Bacilli attacking various carbohydrates with the production of acid and gas.

TABLE III

*Biochemical reactions of Gram-negative bacilli, group 4*  
(sub-groups 1-4)

Sub-group	Xylose	Glucose	Levulose	Galactose	Lactose	Maltose	Sucrose	Raffinose	Mannitol	Dulcitol	Sorbitol	Inulin	Salicin	Asparagin	Dextrin	Glycerin	Starch	Motility	Indol	Methyl red	Voges-Proskauer	Lead acetate	Nitrate reduction	Gelatin liquefaction	Litmus milk *
1	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	-	AG	-	-	AG	AG	-	+	-	-	+	+	+	A
2	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	-	AG	-	-	AG	AG	-	+	-	-	+	+	+	B
3	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	AG	-	AG	-	-	AG	AG	-	+	-	-	+	+	+	A
4	AG	AG	AG	AG	AG	AG	-	AG	AG	AG	AG	-	AG	-	-	AG	AG	-	+	-	-	+	+	+	A

\* BC Indicates that milk was bleached and clotted; AC Indicates acid and clot.

All the cultures for the sugar fermentation reactions were sown into 1 per cent. sugar-peptone-water media and incubated at 20° C. for 21 days before the final readings were made.

The primary anaerobic cultures were plated on Lemco agar after 4 days' incubation and the plates then incubated at 20° C. in the anaerobic jar. Eight cultures were finally selected for further study on the basis of their growth and colony characters. These were all motile, Gram-positive bacilli, which formed subterminal oval spores and produced slight blackening in cooked-meat broth. No other types of Clostridia were encountered. Two of these eight strains gave identical biochemical reactions; the reactions of the seven strains are set out in table IV.

From this table it would appear that these Clostridia may be separated into three ill-defined groups.

In addition to the bacilli described, strains of *Bacillus subtilis* and *Bacillus megatherium* were cultivated from each of the roaches examined.

TABLE IV

Biochemical reactions of seven strains of *Clostridia*

Gelatin liquefaction	Digestion of serum	Litmus milk	Glucose	Lactose	Maltose	Sucrose	Mannitol	Salicin
-	-	-	AG	-	-	-	-	-
-	+	-	AG	-	-	-	-	-
+	+	-	AG	-	-	-	-	-
-	-	-	AG	-	AG	-	-	-
-	-	Digestion	AG	-	AG	-	-	-
-	-	Digestion	-	-	-	-	-	-
-	+	-	-	-	-	-	-	-

Cocci were present only in small numbers, but a white, non-hæmolytic Gram-positive micrococcus which did not produce coagulase was cultivated from every insect, and two strains of streptococci were constantly recovered. Neither of these produced hæmolysin. One corresponded to *Streptococcus non-hæmolyticus* II of Holman's classification; the other to *Streptococcus faecalis*.

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#### Summary

A study of the flora of the alimentary tract of the American cockroach has not demonstrated any bacteria belonging to any of the recognised groups of human pathogens. The flora shows a predominance of Gram-negative bacilli, which have been arranged in four groups on the basis of their biochemical activities.

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618.12—002.7

## FALLOPIAN TUBE GRANULOMA

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Under the title of siliceous granuloma several cases have been reported in which histological changes characteristic of talc granulomata elsewhere have been demonstrated in the Fallopian tubes. The similarity of the histological changes has led to the belief that these granulomata originate from the use of surgical talc in previous abdominal operations. The following case presents the typical features of talc granuloma of the Fallopian tube yet in this instance the introduction of an exogenous irritant can be reasonably excluded. It is considered that this case raises some doubt as to the correctness of attributing these Fallopian tube granulomata wholly to the previous use of surgical talc.

*Case history*

Mrs H., aged 28, married 3 years, a housewife with no children, was admitted to hospital on 12.4.47 complaining of occasional para-umbilical abdominal pain for one year. She also complained of sterility. She had had measles, scarlet fever and whooping cough in childhood. There was nothing relevant in her family history and no tuberculous history, and her husband was fit and well. X-ray of husband's chest normal.

One year before admission she had had an attack of abdominal pain which lasted half-an-hour. She was quite well during the ensuing six months but then suffered a similar attack. Her third attack was six weeks before admission and since then she had had three further attacks each lasting four hours. The pain was localised to the centre of the abdomen and was usually of a colicky nature. Vomiting occurred after each attack. On one occasion the attack was followed by a short period of diarrhoea, but bowel action was otherwise normal. During the first attack there was some scalding on micturition; otherwise there were no urinary symptoms.

Menses commenced at the age of 13 and were always regular except for a two-month period of amenorrhoea at the age of 16. Menses have lasted five days and occurred at regular 28-day intervals except for the last two periods which lasted ten days each. The abdominal pain was not related to menstruation. Apart from slight dysmenorrhoea the menstrual history was normal.

General physical examination revealed some tenderness just below the umbilicus and towards the right iliac fossa. Vaginal examination was normal. X-ray examination of chest and bones showed normal appearances. Blood count and sedimentation rate showed no significant abnormalities apart from a slightly raised lymphocyte count—52 per cent. of 7200 cells. The urine was normal.

At operation on 14.4.47. Sub-umbilical mid-line incision. Masses of small, dark-coloured tumours were found scattered over the pouch of Douglas, the

posterior surface of the uterus, both ovaries and the anterior abdominal wall. The right Fallopian tube was much thickened and there was some doubtful thickening of the left tube. The lesion was considered to be either tuberculosis or endometriosis. The right Fallopian tube was removed. Now, 17 months later, the patient is very well and has had no recurrence of symptoms. One year after the original operation curettage of the uterus was performed as a diagnostic aid, histology revealing a normal post-ovulatory endometrium.

#### *Histology of Fallopian tube and of a peritoneal nodule*

Histological examination of the excised tube shows much thickening of the villi and infiltration with chronic inflammatory cells. The inflammatory reaction is nodular in type and closely resembles tuberculous granulation tissue, but caseation is absent from the follicles. Moreover there are plentiful giant cells containing quite large foreign bodies which, under the polarising microscope, are seen to be doubly refracting.

The peritoneal nodule shows a similar histological picture of "non-caseating tubercle," but the optically active particles are much fewer.

Tubercle bacilli cannot be demonstrated in either site.

#### *Discussion*

The occurrence of this type of Fallopian tube granuloma has recently been reported by several workers. Roberts (1946-47), after reviewing the literature on the subject, described five cases in patients who had been previously subjected to abdominal operations. German (1943), in a review of 40 cases of intra-abdominal talc granulomata, recorded 9 in which the tubes were affected. Smith (1948) recorded a further case co-existing with carcinoma of the uterus. In all these cases there was an antecedent history of an abdominal operation. In the cases of Roberts and of Smith these operations had all been appendicectomies.

The ability of siliceous particles to evoke a foreign body reaction is well established. Shattock (1916-17) was the first to describe a foreign body granuloma resulting from the introduction of silica into the subcutaneous tissue. Faulds (1935) placed the occurrence beyond doubt, establishing the nature of the foreign body by petrological microscopy and microchemical analysis. By this means he was also able to demonstrate the relationship between the foreign body in the tissues and the slate which was responsible for the original injury.

Several other workers have recorded siliceous granulomata in other parts of the body (Antopol, 1933; Antopol and Robbins, 1937; German, 1940, 1943; Sxaen and Tuovinen, 1947), and experimental evidence has been produced by them in support of the siliceous nature of the causative agent.

However, in view of the occurrence of a Fallopian tube lesion of this kind in a patient who had never undergone a previous operation, the invariably siliceous nature of these tubal granulomata is open to doubt. Our patient's original complaints were of abdominal pain and sterility. She denied ever having employed contraceptive measures of any kind and indeed her husband had recently undergone a seminal examination in an effort to determine the cause of the infertility. She also denied ever having used any type of vaginal douche or other similar application, nor had she had any tubal insufflation. It therefore appears reasonable to assume that in this case the optically active particles were endogenous.

The occurrence of endogenous optically active particles is not unusual in routine histopathology. Stewart (1920) showed that even certain normal tissues are optically active (collagen for example) and by the frequent use of

the polarising microscope we wholly agree with his findings. Moreover we have not infrequently found optically active particles in typically tuberculous lesions. The occurrence of these particles in sarcoidosis is well known and through the courtesy of Dr F. B. Smith we have recently been able to examine sections from an unusual case of generalised visceral sarcoidosis in which the Fallopian tubes were involved. They showed a remarkable similarity to our own case of tubal granuloma.

It appears to us that the evidence for the siliceous nature of these granulomata has relied in most cases upon their close histological similarity to undoubted talc granulomata and that until now no example of the condition has been reported in any patient in whom exposure to silica could be reasonably excluded. Apart altogether from the present instance there is evidence to suggest that the siliceous origin of these tubal granulomata is doubtful. In most cases both tubes have been equally involved. In another case, following appendicectomy, the left tube only is described as showing the lesion (Roberts). It is difficult to imagine why the distribution should be so uniform if it depended only upon the haphazard introduction of an unknown amount of talc into the right iliac fossa. It would be more reasonable to assume that the right iliac fossa, being the site most heavily bombarded, would show maximal lesions. Such has not been found to be the case. The lesion is usually distributed fairly evenly over both tubes and the surrounding peritoneum. Nor is this distribution encountered in experimental work of this nature, which shows that the maximal lesions tend to occur at the site of maximal peritoneal injury, while foreign bodies introduced intra-peritoneally have not been observed to lodge in the Fallopian tubes of experimental animals.

The fact that the lesions have always been discovered after previous operation does not justify the assumption that they are caused in this way. Not only is the total number of cases too small to warrant drawing statistically significant conclusions of a direct relationship, but it might even be argued that the tubal granuloma may itself have been responsible for the initial abdominal symptoms. In support of this argument German (1943), in presenting 50 cases of post-operative talc granulomata, observes that some of the patients came complaining of the same symptoms for which they had originally sought relief.

In the 6 cases reported by Roberts and Smith all had undergone previous appendicectomy from five months to 17 years previously, but no pathological evidence of appendicitis was offered. Appendicectomy is often resorted to in an attempt to deal with ill-defined abdominal pain and it is not impossible that some of these appendices were scapegoats for a pre-existing tubal granuloma.

We consider that the evidence for the siliceous origin of this tubal granulomatous lesion is at present unconvincing and prefer to regard it as probably representing an atypical tuberculous reaction of the sarcoid type. In support of this view it may be mentioned that one of the cases cited by German (1943) showed a combination of dusting powder granuloma and proved tuberculous salpingitis and peritonitis, the tuberculous and siliceous lesions co-existing in the same microscopic field.

### *Summary*

A case of tubal granuloma presenting the typical histological features of a talc granuloma is reported in a woman in whom the introduction of talc or other extraneous matter could reasonably be excluded.

It is considered that the assumption of a talc origin in all previously reported tubal granulomata is open to doubt. The occurrence of obviously endogenous optically active particles in sites not exposed to irritation by silica is stressed.

It is believed that these tubal granulomata may in some instances be an expression of an atypical tuberculous reaction of sarcoid type.

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578.67:616—018.74

A METHOD FOR THE DEMONSTRATION OF RETICULO-  
ENDOTHELIAL CELLS IN PARAFFIN SECTIONS

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(PLATE XCI)

The specific demonstration of the reticulo-endothelial system by the use of silver impregnation techniques has been neglected in general pathology, although such methods for the staining of the microglia have been in general use among neuropathologists for many years. In the techniques previously described the use of frozen, or less often of celloidin, sections has been obligatory. The employment of paraffin sections would result in a considerable increase in the application of such methods to routine surgical biopsy material, and in particular to their use for research purposes on post-mortem tissues of which only material in paraffin block is available. The technique to be described, which is a modification of the Weil-Davenport (1933) method, provides staining of a quality equal to the frozen section methods and is also suitable for post-mortem tissues which have been many years in wax. Successful results have been obtained twenty years after the embedding of the tissues. The method of Loughlin (1935) appears to be the only other published technique applicable to paraffin material. This method, however, requires several days for its completion.

*Method*

1. Cut paraffin sections of formalin-fixed material at 15-20  $\mu$  and, without mounting on slides, transfer direct to two successive baths of xylol.
2. On removal of the wax place the sections first in absolute alcohol and then in 50 per cent. alcohol.
3. Wash in two changes of distilled water.
4. Place in the following silver solution for 5-10 seconds. Add, with shaking, 10 per cent. silver nitrate to 2 c.c. of 0.880 S.G. ammonia until a slight permanent turbidity forms (about 18 c.c. of silver nitrate are usually required).
5. Transfer to a 3 per cent. solution of formalin in distilled water for 2-3 minutes, moving the sections in the bath during the reduction.
6. Wash in distilled water. Tone in 0.5 per cent. gold chloride, wash, fix in 5 per cent. sodium thiosulphate, dehydrate and mount in balsam.

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\* In receipt of a whole-time personal grant from the Medical Research Council.

The following points should be observed :

1. If the sections are left too long in the silver solution the method may fail completely : 7-8 seconds is usually the optimum time.

2. The intensity of the impregnation depends largely on the strength of the reducing solution. Too strong a formalin solution results in feeble impregnation, the sections being a pale yellow colour. For the best results the sections should be golden-brown at the completion of the reduction. The strength of the reducing bath may have to be varied to obtain this and it may also require renewal after a number of sections have been passed through it. Each section should accordingly be examined under the low power of the microscope to check the effects of the stain.

The above method will give satisfactory results with the bulk of material studied. The two modifications hereafter described will, however, add greatly to its value in special cases.

#### *Modification 1 : double impregnation*

If the material studied is refractory to the stain or if a specially intense impregnation is required, the following procedure may be adopted :

1. After reduction in formalin (stage 5), wash in distilled water.

2. Transfer for 5-10 seconds to a diluted silver bath composed of distilled water 10 c.c., silver solution as in stage 4, 3 c.c.

3. Reduce again in the original formalin solution and proceed as in the first method.

An extremely intense stain will be obtained from this procedure. Its degree may be controlled by the time spent in the diluted silver solution and by the intensity of the stain before it is placed in this solution. The formalin bath accordingly may have to be strengthened before the impregnation is satisfactory.

#### *Modification 2 : nuclear staining of unimpregnated cells*

In many of the preparations the staining of nuclei of the cells not impregnated by silver is feeble or absent. It is however frequently as important to identify the cells which are unimpregnated as those that are fully stained. The following modification will ensure this.

1. After reduction in formalin, wash the sections in two changes of distilled water.

2. Place for 1 minute in del Río-Hortega's strong silver carbonate solution diluted to half strength with distilled water. (For details of preparing this solution see Russell, 1939).

3. Transfer direct to a 10 per cent. formalin bath, agitating the section in the solution.

4. Wash, tone in gold chloride, fix and mount.

The nuclei will usually become stained after one immersion in the silver bath, but if necessary the sections may be washed in distilled water and put through the process a second time.

#### *Results*

By the use of the methods described the cell-body and processes of reticulo-endothelial cells are uniformly impregnated to an even black. In the central nervous system astrocytes and oligodendroglial cells are also impregnated. Reticulum cells in the spleen and lymph nodes are usually impregnated to a pale grey, but may be colourless. Myeloblasts are not impregnated. The bodies of fibroblasts may be stained, but their differentiation from histiocytes is clearly obtained on morphological grounds. Reticulin fibres are usually not impregnated, but occasionally become so in intensely stained preparations. Epithelial elements are not impregnated.

STAINING OF RETICULO-ENDOTHELIUM

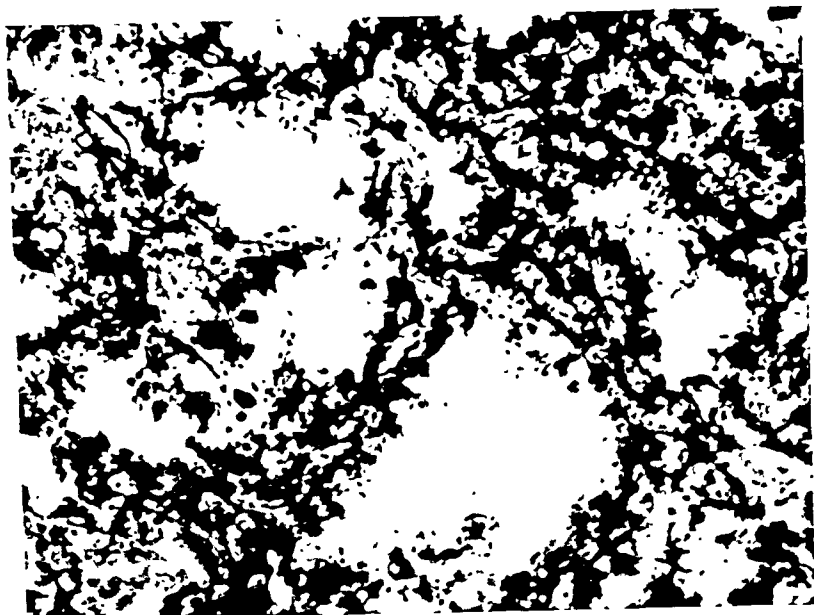


FIG. 1.—Reticulo-endothelial cells surrounding sinuses in pulp of rabbit's spleen  $\times 335$ .

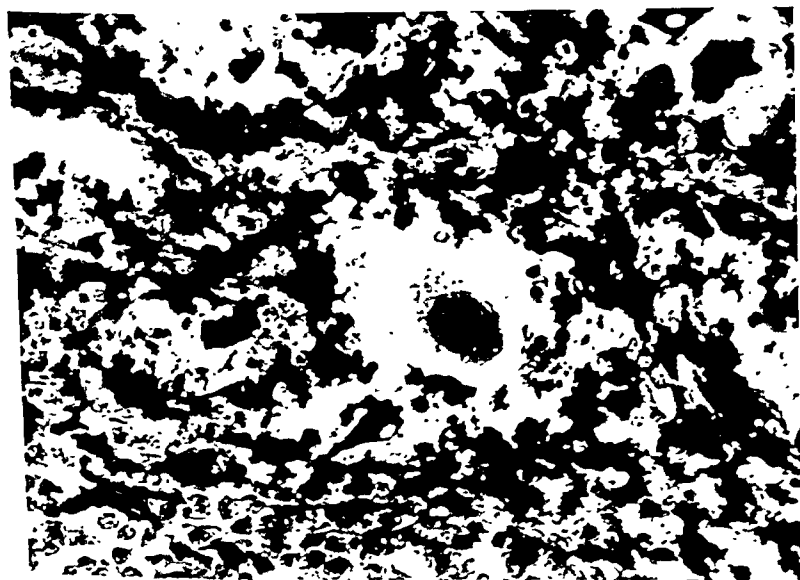


FIG. 2.—Similar preparation counter-stained with silver sodium carbonate to show nuclei of unimpregnated cells. A megakaryocyte is present in the lumen of the sinus and a number of lymphocytes are seen at the bottom of the field.  $\times 510$ .





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EFFECT OF CASTRATION UPON THE INDUCTION OF MAMMARY TUMOURS BY OESTROGEN IN MALE MICE OF THE STRONG A STRAIN

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The incidence of mammary cancer in oestrogen-treated male mice of various strains has usually been found to bear a relation to the spontaneous incidence in non-treated females of the same strains. When Strong A males were treated with oestrogens, however, the observed mammary-cancer incidence was low (Bonser, 1936, 1942, 1944; Bonser and Robson, 1940), although in breeding females living to twelve months or more it was 50 per cent. (Bonser, 1940). The milk factor is known to be present in the milk of Strong A nursing females (Bonser, 1944). The explanation of the low incidence in oestrogen-treated Strong A males was sought in the excess of androgen produced by hyperplastic or neoplastic interstitial cells of the testis. This view was supported by the observation that the pelvic organs of these mice failed to show the usual squamous metaplasia induced by oestrogens. The potency and type of the milk factor was also considered and the present experiments were undertaken with a view to observing the influence on oestrogen-treated males of castration combined with administration of milk factor from the RIII strain, in which the mammary-cancer incidence is high in both breeding females and oestrogen-treated males.

*Experimental procedure*

Two groups of Strong A male mice were used, one castrated, the other not. The spontaneous mammary-cancer incidence in breeding females during the last six years (1942-48) has been 44 per cent. in mice living twelve months or more. Both experimental groups were suckled by RIII foster mothers derived from a strain in which the spontaneous breast-tumour incidence is 84.6 per cent. in breeding females surviving for more than twelve months. That the males of this strain respond satisfactorily to oestrogen is shown by the fact that 62.5 per cent. of 24 male mice treated for twenty weeks or more developed mammary cancer (Bonser and Robson, 1940). The average interval between birth and transference to foster parents was 13 hours for the castrated and 9.3 hours for the non-castrated group. The average age at castration was 5.6 weeks; treatment was started at an average age of 6.5 and 5.5 weeks respectively.

All mice received 3 mg. of triphenylethylene in arachis oil by subcutaneous injection once a week up to the time of death.

*Results*

Twenty-three intact mice suckled by RIII foster mothers (group I in the table) lived to receive treatment for 45 or more weeks (range 45-64, average 57.5 weeks). Twenty castrated foster-nursed mice (group II) survived for a like period (range 44-65½ weeks, average 56.9). As the earliest appearance of mammary cancer in a previous experiment was at 43 weeks of œstrone benzoate treatment (Bonser, 1936), the ranges shown are taken as covering the tumour age.

TABLE

*Interrelation of the type of milk factor and the amount of androgen available and the occurrence of mammary carcinoma*

Experiment	Group	Type of milk factor supplied	State of the interstitial cells of the testis	Amount of androgen available as estimated by the condition of the pelvic organs	Mammary-cancer incidence in mice living to tumour age (43 weeks)	Significance of difference
Present	I	RIII	Benign and malignant tumours	++	0/23 (0 per cent.)	Not significant
	II	RIII	Removed	—	2/20 (10 per cent.)	
Previous (Bonser, 1944)	A	Strong A	Hyperplasia or large tumours	++	1/21	Not significant
	B	RIII	Hyperplasia or large tumours	++	0/8	
	C	Strong A	Removed	—	3/16 (18.8 per cent.)	
	Non-castrated		Hyperplasia, benign and malignant tumours	++	1/52 (1.9 per cent.)	
Combined	Castrated		Removed	—	5/36 (13.9 per cent.)	Just significant

No mammary tumours occurred in group I, but two tumours (10 per cent.) occurred in group II at 51 and 62 weeks respectively. This difference is not statistically significant. When, however, the results of previous experiments are taken into account (Bonser, 1944) and all castrated Strong A males treated with triphenylethylene are grouped together, the incidence is 5/36 compared with 1/52 in non-castrated mice. The increase due to castration is thus 12 per cent.  $\pm 5.8$ , which reaches the lowest conventional level of statistical significance.

When the effect of foster nursing on castrated and non-castrated groups is examined (table), the incidence of mammary cancer in the animals foster-nursed with the more potent milk factor is not increased.

The histological changes in the breast are entirely comparable in groups I and II (13 and 16 breasts respectively examined), and consist in the development of numerous ducts, mainly non-dilated and inactive, with occasional localised areas of acinar proliferation. Dilated ducts were present in four of group I and two of group II mice. Castration appears to have had little or no influence on the growth of the mammary tissue.

Testicular tumours developed in 21 group-I mice out of 23 receiving 43 or more weeks of treatment; of these, two were adenomata, the remainder interstitial-cell carcinomata.

The pelvic organs showed a striking difference. In group I (15 mice examined) no squamous metaplasia was observed in the coagulating gland, which showed naked-eye atrophy in 7. In group II (15 examined) squamous metaplasia was present in all, being extensive in three (61-64 weeks of treatment) and associated with keratinisation in four (56-59 weeks).

### Discussion

It was anticipated that castration of male mice of the Strong A strain, which respond to oestrogen by a high incidence of testicular and a low incidence of mammary cancer, would raise the incidence of mammary cancer by removing the testicular source of androgen. In previous experiments, a slightly increased incidence (not statistically significant) followed castration, but in the present experiment no greater increase was obtained, in spite of the administration of a potent (RIII) milk factor, which might have been expected to enhance the effect. It is to be noted, however, that in all the experiments the tendency has been towards a higher incidence of mammary cancer in the castrated groups and, when the results are combined, the increase just attains statistical significance. In both experiments, castration was followed by a full oestrogenic reaction in the pelvic organs, a fact indicating that the testicular source of androgen is physiologically active and might be expected to manifest its activity on both breast and pelvic organs.

The explanation of the discrepancy between the effect of castration on the mammary gland and on the pelvic organs must therefore be sought in (a) a genetic difference in the mammary gland itself, or (b) a genetic dimorphism in the pituitary gland.

### Summary

Prolonged oestrogen treatment of Strong A males resulted in a high incidence of interstitial-cell tumours and a low incidence of mammary cancer (Bonser, 1944). The excess of androgen produced in this way protected the pelvic organs against the squamous metaplasia which normally results from oestrogen treatment. Castration of Strong A males prior to oestrogen treatment resulted in a slightly increased incidence of mammary cancer, but that the excess of androgen had been removed was shown by the oestrogenic response of the pelvic organs. Even the prior administration of a potent milk factor (RIII) did not increase the mammary-cancer incidence after castration.

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616 . 233—007 . 64 : 619 . 9 (*Rattus*)

## BRONCHIECTASIS IN LABORATORY RATS

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In the course of some tentative experimental work on lung diseases it was found that the stock laboratory rats employed suffered from a spontaneous disease of the lungs which made the experiments useless. The disease, a form of bronchiectasis, was similar to that described by Moise and Smith (1928-29) and by Passey, Leese and Knox (1936), who found an incidence of 51 per cent. in 251 animals studied. Wilens and Sproul (1938) found 75 per cent. of their 487 animals affected.

In the present series, 200 hooded rats of various ages, killed for dissection by a zoology class, showed an incidence of 43.5 per cent. on naked eye examination of the lungs. In its earlier stages the disease causes small grey nodules in the lungs. These progress till whole lobes are occupied by distended bronchi filled with caseous material.

*Pathogenesis*

Bacteriological examination by cultural methods of material from the interior of the bronchiectatic cavities, of mucus from the trachea, and of the bronchial lymph nodes yielded no growth of any organism that seemed to be related to the disease. Most of the cultures were sterile. Nelson (1946 *a* and *b*), however, claimed to have demonstrated the presence of a virus in what he called endemic pneumonia of rats, which, from his description, would appear to be the disease under discussion. He found that it was very common in older rats but that the young were resistant, and that it was not related to the diet, an observation confirming the earlier finding of Passey *et al.* in their studies of bronchiectasis and metaplasia in laboratory rats. Nelson found no bacteria, but could transmit the disease to mice by the nasal instillation of a suspension of lung exudate from affected rats. The active agent was effective in dilutions of  $10^7$ , was removed by centrifuging at 9000 r.p.m. for 30 minutes, but could not be cultivated in embryonated eggs. It remained active for 3 months in the frozen state under dry ice and could be maintained by direct transmission from mouse to mouse but only by the nasal route.

Moise and Smith were inclined to attribute the bronchiectasis to plugging of the bronchi with mucus, after which the growth of organisms occurred. Great accumulation of mucus certainly occurs in the bronchi, but in general, bacteria are conspicuous by their absence. In the present series of animals the accumulation of mucus seems to have been the result rather than the cause of the bronchial obstruction. The cause of the obstruction is believed to be swelling of the mucosal lymphoid tissue, so that polypoidal masses obstruct or distort the bronchial lumen. Mucus accumulates distal to the obstruction and in time causes an inflammatory reaction, for it has been found that even sterile mucus in the lungs of the rat can set up inflammatory changes (Wood, 1941). The combination of obstruction and sepsis provides the conditions which are generally believed to lead to bronchiectasis. The virus isolated by Nelson may well be the cause of the initial enlargement of the bronchial lymphoid tissue.

*Relationship to human bronchiectasis*

In most cases of human bronchiectasis the mechanism here described is probably of little importance. In children, however, enlargement of lymphoid tissue is sometimes very marked, and may then play some part in the aetiology of the disease.

## Summary

In the commonly encountered disease, bronchiectasis of laboratory rats, bronchial obstruction by swollen lymphoid tissue is shown to be an important aetiological factor.

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A NON-GAS-PRODUCING VARIANT OF *BACTERIUM COLI*  
 INDUCED BY GROWTH IN GLYCINE

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Dubos (1946) states that growth in the presence of injurious agents often results in non-specific transmissible modifications of the bacterial cell. He quotes Penfold (1910-11, 1911) as showing that *Bact. coli* loses the ability to produce gas from sugars when grown in monochloroacetic agar. In the course of an experimental study of the effect of amino acids on bacterial growth, Gordon and McLeod (1926) showed that glycine in relatively high concentrations was inhibitory to the growth of organisms. Gordon and Gordon (1943) showed that the colonies of some organisms, particularly *Vibrio cholerae*, were sticky when grown on solid media containing sub-lethal doses of glycine, and stained films made from such colonies showed marked morphological changes. Gordon and Gordon (1947) showed that repeated subculture of *Shigella shigae* in broth containing increasing concentrations of glycine enabled the organism to develop an increasing tolerance to concentrations of glycine previously lethal.

Work on the acquired resistance to glycine was extended to a strain of *Bact. coli* kept in the laboratory. In broth containing glycine its growth was markedly inhibited at a concentration of 1 per cent. and usually prevented at a concentration of 1.5 to 2 per cent. As with *Sh. shigae*, repeated subculture in gradually increasing concentrations of glycine readily increased the resistance of the organism until, after subculture over a period of six months, it grew in a concentration of 6 per cent. glycine in peptone broth. Stained films of subcultures of this organism grown on heated blood-agar showed that, like the cholera vibrio, it now displayed the most extraordinary pleomorphic forms. The reactions of the modified organism and of a subculture of the original strain of *Bact. coli* were then tested on the usual 1 per cent. peptone sugars and in peptone water for production of indole. The sugars, which were incubated for a fortnight at 37° C., were read at intervals, and it was found that both strains produced indole and formed acid in glucose, mannitol, lactose, and dulcitol but not in sucrose. As usual, the original strain produced gas in the

fermented sugars, but the glycine-resistant strain failed to do so even when subcultured on ordinary blood-agar every few days for several weeks.

In addition the cultures in glucose, mannitol and lactose were also subcultured, each into the same sugar, but gas was not produced even after twelve subcultures. This indicates that even continued growth in the sugar does not restore the enzymes responsible for producing gas.

Grey (1913-14), using Penfold's method of developing a strain of *Bact. coli* which did not produce gas from mannitol or glucose, found it also unable to decompose formates. In this connection, Stephenson (1930) concluded that it was highly probable that the hydrogen and carbon dioxide arising in fermentation by *Bact. coli* originated from formic acid or its immediate precursor. The work of Stephenson and Stickland (1932) confirmed the importance of decomposition of formic acid as a source of hydrogen in bacterial fermentations.

That my glycine-resistant variant did not produce gas from sodium formate was shown by the following experiment kindly performed for me by Dr Stickland. Cultures of the glycine-resistant and of the original strain of *Bact. coli* were grown for 36 hours in 0.5 per cent. sodium formate broth. They were centrifuged and after the deposits had been washed with saline suspensions were placed in Barcroft manometer vessels. Sodium formate 0.1 *M* and phosphate buffer at pH 7.0 were added and after the vessels had been filled with pure nitrogen the rate of hydrogen evolution was followed as by Stephenson and Stickland. The glycine-resistant variant failed to form gas whereas the original strain produced it in quantity.

It is interesting and possibly may be of some significance that monochloroacetic acid as used by Penfold is closely related chemically to glycine.

### Summary

A strain of *Bact. coli* grown in increasing concentrations of the amino acid became resistant to 6 per cent. glycine.

The resistant variant still produced acid in glucose, mannitol, lactose and dulcitol, but completely failed to form gas. Repeated subculture in these peptone sugars did not restore the power to produce gas. This finding is probably closely associated with the suppression of the ability to decompose formic acid. The production of indole was unaffected.

I should like to thank the Medical Research Council for a grant in aid of expenses.

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## BOOKS RECEIVED

### Submicroscopic morphology of protoplasm and its derivatives

By A. FREY-WYSSLING. 1948. New York, Amsterdam, London, Brussels : Elsevier Publishing Co., Inc. Pp. viii and 255 ; 160 text figs. 32s. 6d.

This book is concerned with a subject which is fundamental to all biological studies, namely the structure of protoplasm. Its scope is remarkably wide and no doubt other volumes will be written on the same subject before its scope is appreciably extended. The obvious extension, as the author recognises, is in the rapidly advancing field of electron microscopy. Those who set out to interpret electron micrograms will need to understand the principles of structure which are considered in this monograph.

The book is a set of studies in outline, each of which requires much more space for real exposition. But in one volume, closely printed, the author has collected a great number of facts ; the book makes reference to and summarises many interesting studies that would take much time to read and digest from original papers. The author is a pioneer and a foremost Continental worker in this field, particularly its botanical aspects.

The translation into English of certain technical terms is most unsatisfactory, and the degree to which the book is thus marred demands that similar translations should be corrected by an English-speaking expert in the same field. The book is elaborately supplied with clear diagrams, though some of the few electron micrographs are poorly reproduced.

### The leptospiroses

By P. H. VAN THIEL. 1948. Leiden : Universitaire Pers Leiden. Pp. x and 231 ; 19 figs. on 9 plates and 5 text figs. Guilders 16.50.

The Dutch, by their investigations in Holland and the East Indies, have done more work on the leptospiroses than any other nation, and the monograph of Dr B. Waelch-Sorgdrager of Amsterdam has been the standard work on these diseases since its publication in 1939. Professor P. H. van Thiel of Leyden University has written the book under review after participating for many years in the investigation of the subject, and he has given us an account of the leptospiral diseases of man and animals which is very valuable—in Great Britain especially so at the present time, because infections by *L. canicola* have been more frequently recognised this year and last than ever before.

In this book, the morphology of the leptospiræ is well described from examination by the electron microscope, carried out by the author and others. The opinion is expressed that these organisms are so much akin to both bacteria and protozoa as to form an intermediate or transitional family. Their antigenic stability, the relation of the different species to one another and the separation of sub-species with incomplete antigenic structure have been discovered by the serological tests of agglutination and lysis and by absorption of antibodies from serum. These methods, with the results obtained, are given broadly.

The technique of diagnosis, in animals and man, of both active and previous leptospiral infections is well and fully described and provides one of the most valuable chapters in the book. The advantage for dark-ground microscopy and culture of concentrating leptospiræ by centrifuging oxalated blood is emphasised. Methods of serological examination are carefully described, though some confusion is caused by the use of the term "weakest dilution," where "lowest dilution" is intended.



The general summary of epidemiology, including the role of rats, voles, dogs and other animals as carriers of various species of leptospiræ, is well done but, as in some other chapters, there are errors which should be corrected in any future edition. For instance on p. 66 it is stated that significant antibodies have been found in miners who had been exposed to infection, but on p. 96 the same work is accurately referred to as a finding in sewer-labourers. In a chapter on general and special prophylaxis the need for disinfecting patients' urine and for rubber gloves to be worn by nurses attending patients is emphasised. These precautions are not usually taken in this country. A chapter on clinical features and treatment, especially of leptospirosis ictero-hæmorrhagica, is useful but rather vague. A mistake is made in reporting the work of Alston and Broom (*Brit. Med. J.*, 1944, ii, 718) on the effect of penicillin on leptospiræ *in vitro*; they found, in fact, that *L. ictero-hæmorrhagica* and *L. canicola* are clearly sensitive to penicillin.

Almost half of the book is occupied by accounts of thirteen species of leptospiræ which have been found to infect man and animals and of another eight species known to infect animals but not man. These very valuable chapters bring together knowledge not collected elsewhere, but they contain some regrettable omissions and mistakes. For instance, regarding leptospirosis canicularis, the reported cases of human infection are stated (p. 138) to number 25 and to have occurred in Holland and Denmark. But Dr Borg Petersen of Copenhagen reported 47 cases in Denmark alone up to 1943, Dr Minkenhof of Amsterdam in 1947 reported a total of 49 in Holland and well-authenticated examples were reported before 1947 in the U.S.A., Great Britain, France, China, Switzerland and Germany. On p. 181, in the chapter on leptospirosis schüffneri, it is stated that "Buchanan (1927) records that he has observed in a guinea-pig, which was inoculated with renal tissue of a bat, typical symptoms of Weil's disease." "Bat" is a mistake, probably due to a mistranslation of "field-mouse," this being the animal on which Buchanan made his observation. The translation into English shows many other mistakes.

There is a full bibliography and an index of authors, but no subject index.

The book is valuable for its wide scope but in its present state it is not a safe guide for those unfamiliar with the subject. It is to be hoped that in a second edition mistakes and omissions will be remedied.

#### Diagnostic procedures for virus and rickettsial diseases

1948. New York: American Public Health Association. Pp. vii and 347; 20 text figs. \$4.

For pathologists interested in virus work of a diagnostic or research nature a manual of laboratory techniques has been lacking; the present publication is designed to meet this need. Each of the sixteen sections in the book has been written by one or more members of a special committee of the American Public Health Association. The subjects dealt with are psittacosis, lymphogranuloma venereum, trachoma, inclusion blennorrhœa, variola and vaccinia, influenza, primary atypical pneumonia, mumps, poliomyelitis, encephalitis, rabies, herpes simplex, yellow fever, dengue, phlebotomous fever and rickettsial diseases. The general plan followed by most of the authors has been an introductory section of varying length, details of the methods used for isolating and identifying the causal agent, and a description of serological methods used in diagnosis. A useful list of references is appended to each chapter. In some instances this plan is of necessity modified: in the chapter on primary atypical pneumonia, methods for the isolation of virus are obviously lacking and serological

methods of diagnosis are not of value in trachoma and poliomyelitis. It is stated in the preface by Francis, chairman of the committee, that "great effort has not been made, editorially, to obtain uniformity in the detail of presentation or wording." As each chapter has been written by an authority experienced in investigation of the disease under consideration, the particular methods and techniques described are those favoured by the individual authors. The procedure for the complement-fixation test used in the examination of vesicle fluid and crusts from suspected smallpox cases as outlined on page 91 appears to the reviewer to be less satisfactory than that of Craigie and Wishart (*Canad. Publ. Hlth. J.*, 1936, xxvii, 371). There is a good deal of repetition in the descriptions of particular techniques, as might be expected in a composite work of this kind; for example the technique for complement fixation tests is described in several chapters with variations only in detail. In future editions additional cross references between individual chapters in such matters might effect some economy in space. The treatment in some sections is much more elaborate than others. For example the first section on psittacosis extends to 45 pages and includes an introduction of six pages touching on epidemiological features, clinical manifestations, pathological lesions and special characteristics of the virus, while the chapter on variola and vaccinia occupies only 10 pages, devoted almost entirely to technical methods. The procedures described in the section on primary atypical pneumonia are those for the detection of cold hæmagglutinins and agglutinins to streptococcus M.G. In a book which contains a short chapter on phlebotomous fever, the investigation of which involves the use of human volunteers, it seems surprising that no space has been found for infective hepatitis, measles, chickenpox or zoster. The illustrations are good and at the end of the book there is, in addition to a general index, an index of tables, charts and illustrations. Notwithstanding the minor criticisms implied in these comments this book will be welcomed by those interested in laboratory techniques for the investigation of virus and rickettsial infections; it supplies a much-needed collection of reliable information in this field.

#### Gynæcological histology

By JOSEPHINE BARNES. 1948. London: Harvey and Blythe. Pp. xii and 242; 162 figs. 30s.

This volume, although it hardly sustains the claim on the jacket ("a new Teacher") is a good, straightforward book. On first examination it is rather disappointing, because one is left in a state of tranquillity quite alien to a subject so "alive" as gynæcological pathology. Theories, perplexities and matters of contention are alike smoothed over in a manner that seems even ingratiating at the time. But if one has read the preface before examining the text (and the present reviewer allowed his zeal to prevent this simple procedure) one will find that this merely indicates an intention well achieved, the realisation of a set purpose; for in the preface we are told that a dogmatic statement has often done duty for a résumé of controversial views in order to keep the book as clear and simple as possible.

Within these limitations, then, the book is a good one. It will be much appreciated by students and recent graduates intending to specialise; the former are amply accommodated, the latter will take the hurdles as they are met, being in that delightful atmosphere where difficulties can be debated and profounder questions answered, or at least canvassed, by reference to profounder literature.

The book contains a higher ratio of illustrations to text than one has ever seen in anything other than a mere atlas, but since the writer's intention

is descriptive rather than controversial, this may well represent an advantageous modern trend. Her pictures are usually very good, sometimes exceptionally so. Only very occasionally are they poor—fig. 16 for example, which, in the reviewer's opinion, fails entirely in its purpose.

### Biology of pathogenic fungi

Edited by WALTER J. NICKERSON. 1947. Waltham, Mass.: The Chronica Botanica Co.; London: Wm. Dawson and Sons, Ltd. Pp. xx and 236; frontispiece, 46 figs. on 8 plates and 30 text figs. \$5.

It might have been thought that several recent additions to the literature of medical mycology, written from various view-points, covered all the needs of microbiologists interested in this discipline, but it rested with Dr Nickerson to discover a hiatus and to fill it with his admirable "Biology of pathogenic fungi." The editor and principal contributor is well known for his work on the physiology and especially the respiration of micro-organisms, and his contributions to this subject in the present volume are especially valuable. He is ably supported by a group of contributors each of whom is a recognised expert in the subject on which he writes. J. Lodder and A. de Minjer deal with the biology of the pathogenic yeasts which cause torulosis and A. J. Carrion and M. Silva with the fungi of chromoblastomycosis. R. Benham deals with *Pityrosporum ovale*, a physiologically interesting if pathologically unimportant yeast, and C. W. Emmons with *Coccidioides immitis*, to our knowledge of the life history of which he has himself made substantial contributions. R. Ciferri and P. Redaelli review the recent important work of the Italian school of mycopathology, which covers the general field of fungi and algae pathogenic for animals and plants, and F. T. Wolf discusses the action of the sulphonamide drugs and natural antibiotics on the fungi. D. S. Martin presents a survey of the geographical distribution of the systemic mycoses, illustrated with world maps, but in attributing diseases to a country he does not discriminate between the truly indigenous infections and mere casual importations. W. J. Nickerson, in addition to the introductory chapter, has contributed chapters on the metabolic products of fungi, respiration and fermentation by fungi and, jointly with J. W. Williams, a section on nutrition and metabolism. The final contributor is R. L. Peek who deals with fungal lipids and wax-like substances in pathogeny and immune reactions. To every chapter the relevant bibliography is appended and the work is completed by three separate indexes, of authors, micro-organisms and subjects.

The book is well got up and is a valuable addition to the literature of mycology. It should appeal strongly to those specially interested in the physiology of fungi.

### Laboratory technique in biology and medicine

By E. V. COWDRY. 2nd ed., 1948. Baltimore: The Williams and Wilkins Co. (British agents, Baillière, Tindall and Cox.) Pp. vi and 269. 22s.

One must be critical about this second edition, for the errors of the first have been handed on. These are obvious in the naming of Europeans: H. G. Cannon, A. C. Lendrum, E. Meulengracht (not only wrongly spelt but in the wrong place alphabetically for either spelling) and Macchiavello, wrongly spelt in the first edition and altered in this but still short of the mark, Euparal, polystyrene and Quellung are also misspelt. This type of error raises doubts about the accuracy elsewhere, for some of these mistakes are repeated and are obviously not simple printer's errors like the direction for making Jenner's stain where eosin and methylene blue are to be mixed in a "flash." The reference to "the current war" suggests transposition without scrutiny from one edition to the other.

It is intriguing no doubt to find quite a large section on the "Revival of Vinegar Eels," and the medical jurist will be interested to note that this edition has an entry under "Crime Detection Techniques." There are also occasional snippets about woad and similar dyes; thus we are told that saffron was spread on the streets of Rome, but not a word about Masson's erythrosin-saffron method, which is still in use in France. Neither Goormaghtigh nor per-iodic acid is indexed, whereas Scandium and Yttrium are (both incidentally in a valueless way). Information about the keeping qualities of staining solutions would be valuable, so also would reference to such useful laboratory vade-mecums as Langeron's *Précis de microscopie* and the *Manuel technique de microbiologie et de serologie* of Calmette, Boquet and Negre (now Boquet, Negre and Bretey).

Room might well be found by eliminating the dictatorial verbiage, e.g. "Erythrocyte counts do not fall in the scope of this book. It is sufficient to state that they are going out of fashion because of the large experimental error involved," or "Normals, Microscopical. Most tissues are examined in but a cursory way. If something is encountered which looks definitely unusual the question of normality comes up, but there are probably numerous instances of tissues which look enough like what was expected to be passed without comment even though they were not in fact normal."

This book recalls Bishop Butler on the laying out of thoughts which are still not unravelled: "It is coming abroad in disorder, which he ought to be dissatisfied to find himself in at home."

#### **Oxidation-reduction potentials in bacteriology and biochemistry**

By L. F. HEWITT. 5th ed., 1948. London County Council. Pp. 130; 30 text figs. 4s. 6d. (by post 4s. 10d.)

The new (5th) edition of this valuable monograph brings under review the very considerable literature of its subject that has accumulated since the previous edition in 1936. The greater part of the matter is, of course, carried over from the earlier editions, but a number of the sections have been rewritten and extended, e.g. that on cytochrome, and new sections have been introduced to deal with the application of oxidation-reduction potentials to milk grading and to the study of chemotherapy and the antibiotics.

There is also an additional short chapter on the application of polarographic methods to the study of oxidation-reduction potentials. This is scarcely adequate as a guide to those unfamiliar with this technique in its practical application, but does indicate its use in quantitative observations on the concentrations of oxidation-reduction systems present and for observations on such systems as oxygen and hydrogen and their compounds, and also sulphhydryl compounds.

An interesting point brought out is the relationship of the previously observed influence of oxidation-reduction potentials on the persistence of virulence in streptococcal cultures and Elliott's observation of the loss of type-specific characters under the influence of streptococcal proteinase. The assumption is that the ferment works under reducing conditions, i.e. negative O.R. potential, and it was observed previously that virulence was maintained by measures assuring a relatively positive oxidation potential in cultures.

#### **Practical section cutting and staining**

By E. C. CLAYDEN. 1948. London: J. and A. Churchill, Ltd. Pp. vii and 129; 21 text figs. 9s.

This little book by the senior technician in the morbid histology department of the Bland-Sutton Institute of Pathology, the Middlesex Hospital,

is expressly written "for technicians with little or no experience in the various methods of preparing routine sections." Stress is therefore laid on the small details, obvious to the expert, which are essential in the preparation of good sections. The hope is expressed that the book will also be found useful by those preparing for the examinations of the Institute of Medical Laboratory Technology. Since this is one of its principal aims it is natural that in general the more highly specialised and less well known methods are omitted. None the less it should prove, especially for younger workers, a very useful addition to the pathological laboratory bookshelf.

Fixation, embedding and section cutting are first considered and the author deals at length with many of the pitfalls which beset the path of the inexperienced and the unwary. The staining methods which follow are those which have stood the test of time and are adequate for all routine pathological laboratories. They are well set out in a clear and concise matter. The book concludes with a number of the formulæ used in the preparation of the more important reagents required in histological work, and an index of six pages. An admirable little book for quick and handy reference.

### Diagnosis in daily practice

By BENJAMIN V. WHITE and CHARLES F. GESCHICKTER. 1947. Philadelphia, London and Montreal: J. B. Lippincott Company. Pp. xvi and 693; 360 text figs, 12 plates (93 figs.) in colour. \$15.

The purpose of the authors of this book is to lay down a complete diagnostic scheme capable of dealing with anything other than rarities. To establish sense of proportion they commence with a short section on the statistics of mortality and disability from the major diseases, based on the data collected in the National Health Survey of the United States Public Health Service. This is a most valuable section, completed by an entertaining list of "Rare Diseases Overemphasized in Medical Diagnosis" which warmed the reviewer's heart.

There follow three sections, taking up about half the book, dealing respectively with the symptoms, signs and laboratory investigations taken system by system. Each chapter has cross references to the second half of the book, which bears the title "Differential Diagnosis of Major Disease Groups." This is comprehensive, including dermatology and psychiatry, and for the most part follows conventional lines. Great efforts are, however, made to summarise the deductive steps in tables. This method is carried much too far and too rigidly: it is the least useful feature of the book.

There are many photographic illustrations, both in black and white and in colour, most of them excellent. The colour photographs of oral lesions, where, it must be admitted, the technical difficulties from reflections are very great, are a little disappointing. There are in addition numerous line drawings of great value: a striking example is the series showing the distribution of the commoner skin lesions. There are select references to each chapter, almost exclusively to American literature.

This book represents a sustained effort to present a logical system of diagnosis and is full of valuable data and illustrations. The effort must have been of the greatest value to the authors themselves and the book is well worth referring to by any physician in difficulty. It is doubtful, however, whether it will achieve its purpose of becoming an essential daily tool for the general practitioner. The attempt to systematise has been carried too far. In practice, medical knowledge has more often to be applied intuitively, according to art.

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## THE CLINICAL FEATURES AND PATHOGENESIS OF MOUSE-POX (INFECTIOUS ECTROMELIA OF MICE)

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(PLATES XCII-XCIV)

BURNET and Boake (1946) showed that the virus of mouse-pox (infectious ectromelia of mice) was closely related to vaccinia virus. Subsequent studies of the clinical signs and epizootic behaviour of the disease (Fenner, 1948*a*) showed that it resembled the acute exanthemata of man in the relatively long incubation period and in the development of a rash which first became evident some days after the onset of the disease. An understanding of the pathogenesis of the acute exanthemata has been hindered by the lack of suitable susceptible laboratory animals which would allow direct studies of the human diseases to be made. In the absence of these direct studies it was thought that mouse-pox might be used as a model of the exanthemata, and investigations into its pathogenesis were therefore undertaken. This paper describes the macroscopic and histological appearances of the skin lesions of mouse-pox and the pathogenesis of the disease.

### MATERIALS AND METHODS

Two strains of ectromelia virus were used, the "Hampstead" strain of Marchal (1930), which had undergone at least fifty egg passages at various times between 1936 and 1947, and the "Moscow" strain, of which the first egg-passage material was used. Stock virus was prepared from infected chorio-allantoic membranes, and virus titrations were carried out on the chorio-allantois by the pock-counting method (Burnet and Lush, 1936) and also by the intraperitoneal and pad inoculation of mice.

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Antibody levels were estimated by the hæmagglutinin-inhibition test, the ectromelia anti-hæmagglutinin (E-AHA) titres being expressed as the reciprocal of the dilution of serum in 1 per cent. fowl erythrocyte suspension which would permit partial agglutination of the latter by four hæmagglutinating doses of virus.

All mice were bred in the Hall Institute mouse room, and were used when eight weeks old. The stock is heterogenetic and free from serious enzootic diseases.

In the principal experiments upon which the hypothesis of the pathogenesis of mouse-pox is based, 120 mice were inoculated in the pad of the left hind foot with 0.05 ml. of a  $10^{-6}$  suspension of Moscow ectromelia virus containing about 500 infective particles \*; at another time 60 mice were inoculated with about the same amount of the Hampstead strain of virus. At daily intervals two mice in each group were killed by bleeding from the axilla. Serum was saved for antibody titrations, and 0.5 ml. of blood was mixed with a similar volume of sterile citrate saline, namely 2 per cent. sodium citrate ( $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$ ) in 0.9 per cent. NaCl. At certain times additional moribund animals were also killed. With separate sterile instruments for each operation *the inoculated foot, a piece of skin from the abdominal surface about one square centimetre in size, and the spleen were removed.* After thorough grinding of the minced tissues in pestles and mortars with a small quantity of sterile alundum, an approximately 20 per cent. suspension of each was made in normal saline containing 10 per cent. horse serum. To detect low concentrations of virus, 0.5 ml. of the original 20 per cent. suspensions and of a 1 in 100 dilution of them was inoculated intraperitoneally into two mice. Specific lesions in the liver and spleen at death or when the mice were killed 12 days after inoculation, and in the absence of such lesions positive serological tests, were taken as evidence of infection. High dilutions of the suspensions were titrated on the chorio-allantois of 11- or 12-day-old eggs. Hundredfold serial dilutions were made by adding 0.05 ml. to 5 ml. of normal horse serum-saline with a marked Pasteur pipette, which was not washed or changed during the series of dilutions. Two or three eggs were inoculated with 0.05 ml. of each dilution and examined for specific lesions with a plate microscope after three days' incubation at 35° C.

When the mice were killed, a second piece of skin was removed from the abdominal surface and fixed overnight in formol-saline. Sections were subsequently cut and stained with hæmatoxylin and eosin and with Mann's stain (eosin-methylene blue) to demonstrate the cytoplasmic inclusion bodies of ectromelia.

#### *The accuracy and sensitivity of virus titration*

Stone and Burnet (1946) demonstrated that, both in the chorio-allantoic membranes and in the rabbit skin infected with vaccinia virus, the amount of hæmagglutinin is not a reliable index of the virus content. Destruction of hæmagglutinin and its neutralisation by antibody both play a part in the dissociation of hæmagglutinin level from virus concentration. In preliminary experiments with ectromelia virus, similar discrepancies were found and in addition hæmagglutinin could not be detected until the concentration of virus was high (about  $10^8$  ID<sub>50</sub> per g.). Hæmagglutination was not, therefore, a satisfactory technique for determining the mechanism of infection in mouse-pox. Titration by the intraperitoneal or pad inoculation of mice was sensitive and fairly accurate if serial tenfold dilutions of suspension were inoculated into groups of 3 or 4 mice, but it was slow and laborious and required large numbers

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\* Except where the chorio-allantoic level is specifically mentioned, one infective particle = one 50 per cent. infective dose (ID<sub>50</sub>) for the mouse.

of mice when at least eight suspensions of unknown virus content had to be titrated each day. Chorio-allantoic titration is rapid and accurate if six eggs are used at each dilution, but this required more eggs than could be handled. The combination finally adopted was to titrate suspensions at low dilutions (0.5 ml. of the 20 per cent. suspension and a 1 in 100 dilution thereof) by the intraperitoneal inoculation of mice, and prepare hundredfold serial dilutions from  $10^{-1}$  or  $10^{-2}$  to  $10^{-6}$  and titrate them on the chorio-allantois, two or three eggs being used at each dilution.

The accuracy and sensitivity of these methods of titration have been determined and an estimate made of the magnitude of a significant difference between two readings. A suspension of infected membranes (Moscow strain of virus) was carried through serial tenfold dilutions in normal saline containing 10 per cent. horse serum, with a clean pipette for each dilution. Between the tenfold dilutions from  $10^{-2}$  to  $10^{-3}$  twofold dilutions were made to provide concentrations of  $10^{-6.3}$ ,  $10^{-6.6}$ ,  $10^{-7.3}$ ,  $10^{-7.6}$ ,  $10^{-8.3}$ , and  $10^{-8.6}$ . This operation was carried out twice and twelve eggs were inoculated with 0.05 ml. of each  $10^{-6}$  dilution. Six mice were inoculated in the pad of the hind foot with 0.05 ml. of each dilution from  $10^{-6}$  through twofold dilutions to  $10^{-9}$ . The egg membranes were examined with a plate microscope after three days' incubation at  $35^{\circ}$  C. and the pocks counted. Post-mortem examinations, confirmed if necessary by estimation of the E-AHA titre of the serum, were carried out on all surviving mice thirteen days after inoculation.

The virus content of the suspension for the mouse inoculations, estimated by the method of Reed and Muench (1938) was  $10^{10.24}$  infective particles per ml. The mean numbers of pocks counted on replicated chorio-allantoic titration of the  $10^{-6}$  suspensions were  $32.1 \pm 10.4$  and  $31.6 \pm 12.9$ , giving a concentration of  $10^{5.60}$  infective particles per ml. The concentration as determined by the pad inoculation of mice (and in other experiments intraperitoneal inoculation gave closely comparable results) was therefore 1.43 log. units, that is about 27 times higher than that obtained by chorio-allantoic titration. This result is the reverse of that obtained by Burnet and Lush with the Hampstead strain of virus, but other experiments indicated that the difference was due not to the strain of virus used but to the limited criteria of infection of mice available to the earlier workers, namely specific death or post-mortem lesions.

The accuracy and reliability of virus concentrations estimated by serial hundredfold dilutions, using the same marked 0.05 ml. Pasteur pipette throughout without washing, was next determined by making ten series of dilutions from  $10^{-2}$  to  $10^{-6}$  of the same preparation of virus as was used in the previous tests, two eggs being inoculated at each dilution. The pock counts obtained upon each pair of eggs inoculated with the  $10^{-6}$  dilution were averaged and the virus concentration of the suspension was estimated from each set of figures obtained. The ten replicated titrations gave a mean value of  $10^{9.21 \pm 0.20}$  infective particles per ml. Comparing the results obtained in the ten estimations made by this method of dilution and titration it can be seen that a difference of  $2 \times \sqrt{2} \times 0.20$  log. units, that is of 0.56 log. units or a 3.6-fold difference between two readings would occur by chance only once in twenty titrations.

Rapid titration without using clean pipettes for each dilution resulted in an estimate of the virus concentration which was 0.41 log. units higher than that obtained by careful titration on the chorio-allantois. This systematic error is unimportant from the present point of view, but the relationship between chorio-allantoic figures obtained by the "rapid" method of titration and those obtained by the inoculation of mice is important. The latter method is 1.03 log. units or about ten times more sensitive.

To obtain a uniform method of expressing the virus content of the different suspensions tested, the chorio-allantoic figure obtained in the manner described was multiplied by a factor ten to make it comparable with the mouse-inoculation



figure and then by a figure of two (for blood) or five (for tissue suspensions) to compensate for the dilution involved in making the original suspensions. When the mouse inoculations only were positive, the virus content was estimated directly, weight being allotted to death times in deciding interpolations. The final concentration of virus is therefore expressed as the number of infective particles ( $ID_{50}$ ) for the mouse per ml. of blood or per g. of tissue examined.

There was a further source of error of which no experimental estimate could be made, namely the difference due to variable efficiency in grinding the tissues. This was largest for the skin and smallest for the spleen. In view of the regularity of the results obtained it seems not unreasonable to consider that this error was of about the same magnitude as that involved in dilution and titration of the suspensions, so that the total error involved is such that a tenfold difference (1.0 log. units) between two results would be significant at the 95 per cent. level.

### THE CLINICAL FEATURES OF MOUSE-POX

The outstanding clinical features of mouse-pox are the primary lesion and the secondary rash. These were briefly mentioned in previous papers (Fenner, 1947*a*, 1947*b*, 1948*a*) but no detailed description was published of their development or histology. The primary lesion occurs at the site of entry of the virus. The secondary rash is a widespread eruption which may involve the greater part of the body surface. The account which follows is based upon observations made with the Moscow strain of ectromelia virus; the Hampstead strain produces similar but less severe lesions.

#### *The primary lesion*

When 350 normal male mice were exposed to contact infection with Moscow ectromelia virus in the course of several closed-epidemic experiments, infection was first recognised in 80 per cent. (280 animals) by the appearance of the primary lesion, in 12 per cent. by death, and in 8 per cent. by the appearance of the secondary rash. Table I shows the sites upon which primary lesions were found.

TABLE I  
*Sites of primary lesions of mouse-pox (Moscow strain)  
in 280 mice infected by contact*

Head (nose, cheek, angle of jaws, lips)	55 per cent.
Eye	18 "
Foot (fore and hind)	5 "
Belly wall	12 "
Back	10 "

The primary lesion appeared first as a localised swelling, on the upper lip, for example, surmounted by a minute breach of the surface. It rapidly increased in size, and oedema of the surrounding tissues was much more pronounced than in individual lesions of the secondary rash (fig. 1). A hard adherent scab formed over the site of the primary

lesion, and a primary lesion several days old was usually quite prominent, being covered with a thick, dark-brown scab under which healing progressed. After 7-14 days the scab fell off and the site of the primary lesion was then marked by a deep hairless scar which often persisted for the rest of the animal's life.

Histologically, advanced pathological changes were present in the earliest clinically recognisable primary lesions. There was usually no obvious breach of the skin surface, but the dermis and subcutaneous tissue were oedematous and there was widespread and fairly intense lymphocytic infiltration of the dermis. Inclusion bodies were usually visible in the epithelium at the summit of the lesion. Ulceration occurred within the first day after recognition of the primary lesion, and in most cases the primary lesion was not diagnosed until ulceration had occurred. The ulceration was caused by necrosis of the infected epidermal cells, and within the next 24 hours widespread necrosis occurred through the dermis also, involving especially hair follicles and sweat glands. Nuclear debris, pyknotic nuclei and lymphocytes were numerous throughout the dermis, and there were many pus cells in the more superficial part of the lesion and in the scab. Healing occurred during the next few days with replacement of the dermis by fibrous tissue and growth of new epidermis beneath the scab. From the earliest stages of the primary lesion the regional lymph nodes were enlarged and engorged, and on section necrotic foci could usually be found within them.

To investigate the pathogenesis of mouse-pox it was desirable to inoculate a known dose of virus by a route comparable to that involved in the natural disease. Inoculation of 500 infective particles suspended in 0.05 ml. of fluid into the pad of one of the hind feet fulfilled these criteria, and the incubation period and course of the disease after such inoculations were identical with those observed in natural infections (Fenner, 1948*a*). The swelling of the foot was taken as the appearance of the primary lesion.

### *The secondary rash*

The secondary rash was seen in 59 per cent. of 202 mice used in several experiments involving exposure to infection with Moscow ectromelia virus. The majority of the animals in which no rash was seen died of the acute disease, usually 7 or 8 days after their first exposure. A few very early lesions were seen in a number of acutely fatal cases, especially in mice which lived for 9 or 10 days after infection. A few mice which had shown florid eruption of the rash died later, but the great majority survived. Table II shows the proportion of mice in each of these categories among those that died and those that survived infection.

In mice with a severe rash, individual lesions appeared all over the surface of the body; conjunctivitis was almost invariable; ulcers

were common on the tongue but rare on other parts of the buccal mucosa; and lesions were not found on the nasal mucosa. Focal

TABLE II

*The incidence and severity of secondary rashes in 202 mice exposed to infection with mouse-pox (Moscow strain)*

	Fatal cases 130	Survivors 72
No rash . . . . .	62 per cent.	3 per cent.
A few very early lesions . . . . .	30 "	7 "
Moderate rash . . . . .	0 "	39 "
Severe rash . . . . .	8 "	51 "

multiplication of the virus was fairly common in the intestinal epithelium (Greenwood *et al.*, 1936) and was occasionally noted in the kidneys, lungs, pancreas and elsewhere. The fully developed rash is shown in fig. 2.

*Times of appearance of the primary lesion and the secondary rash*

The times of appearance of these lesions were studied in a large number of mice inoculated in the pad of the hind foot with 500 infective particles of either Moscow or Hampstead ectromelia virus. The hair of the abdominal wall was shaved and all the mice were examined daily. Similar time intervals were observed in experiments involving short periods of exposure to contact infection (Fenner, 1948a). The figures (table III) show that the intervals between inoculation of a

TABLE III

*The time relationship of the clinical signs of mouse-pox infection produced by the inoculation of 500 infective particles of virus into the pad of the hind foot*

Time from inoculation until	Moscow strain		Hampstead strain	
	Time (days) $\pm$ SD	Number of observations	Time (days) $\pm$ SD	Number of observations
swelling of the foot . . . . .	7.3 $\pm$ 0.8	70	8.0 $\pm$ 0.8	26
secondary rash . . . . .	9.5 $\pm$ 0.7	38	10.1 $\pm$ 0.7	18
death without secondary rash	8.8 $\pm$ 0.8	34	...	0
Interval between appearance of				
primary lesion and secondary rash	2.1 $\pm$ 0.9	38	1.9 $\pm$ 0.8	18
primary lesion and death without secondary rash	1.8 $\pm$ 0.8	34	...	0

standard dose of virus and the appearance of the primary lesion and secondary rash were relatively constant, and were shorter with

## MOUSE-POX (INFECTIOUS ECTROMELIA)

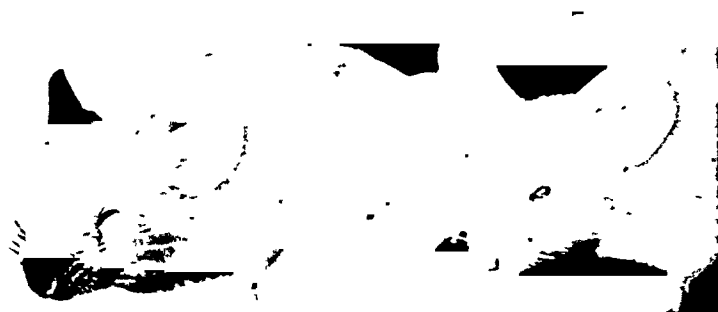


FIG. 1.—An early primary lesion beneath the eye of a mouse exposed to infection with ectromelia virus (Moscow strain) 7 days before. Comparison with the normal mouse on the right shows the swelling of the face due to edema round the primary lesion. A late primary lesion is figured in a previous article (Fenner, 1948a).



FIG. 2.—A moderately severe secondary rash of mouse pox occurring in a mouse exposed 11 days earlier to infection with ectromelia virus (Moscow strain). The skin over the belly wall has been depilated.



the Moscow than with the Hampstead strain. It is also apparent that in fatal cases without a secondary rash the animals died just before the first lesions of the rash usually appeared. It will be shown later that this is why a rash failed to develop in these acutely fatal cases.

## MORBID ANATOMY AND HISTOLOGY

### *The secondary skin lesions*

The appearance and development of these lesions and their histological features were observed in the two experiments from which figs. 3 and 4 were derived. The following description applies to mice inoculated with the Moscow strain of virus. The hair on the bellies of the mice was shaved so that the evolution of the lesions could be carefully observed.

Individual lesions were first seen on the 9th day as slightly raised pale areas of skin two or three mm. in diameter. They increased in number during the next 24 hours, and could then be felt in the skin of the back as small nodules. During the next day the lesions increased in size and new ones appeared. Ulceration began on the 11th day and within the next 24 hours the mature papules were converted into ulcers with adherent scabs by the massive necrosis of the epithelial cells. Healing occurred beneath the scabs, which fell off about the 18th day, leaving hairless scars. Animals which developed severe secondary rashes still showed hairless patches a year after recovery from the infection.

Histologically, no abnormality was seen in sections of skin until the 7th day. A few small localised areas were then noticed in which the epidermis was slightly hyperplastic and the basal cells appeared swollen, with dark-staining nuclei surrounded by vacuoles (fig. 5). In some of these swollen cells cytoplasmic inclusion bodies could be found in sections stained by Mann's method. The dermis and the superficial layer of epidermal cells were normal. The appearance of these early lesions was very similar to that described by Dible and Gleave (1934) in the earliest skin lesions of generalised vaccinia in man. On the next day these areas of proliferation and œdema were much wider, but the greater part of the skin was normal in appearance. Peripheral spread from the original foci had apparently occurred. Inclusion bodies and a few pyknotic nuclei could now be seen in the epidermal cells of the superficial as well as the basal layers and in cells of the adjacent hair follicles and sweat glands. There was still no change in the dermis.

By the 9th day these areas of affected cells had become apparent to the naked eye as pale slightly raised papules. Fresh foci appeared in the intervening areas of previously normal skin and the papules became much more numerous. By the 10th day there was necrosis of the superficial cells of some of the early lesions. There were







are logarithmic and the time intervals are plotted arithmetically. Smooth curves have been drawn through the plotted points with some degree of freedom, for the data were derived from a large number of animals and the problem in which we are interested is the progress of infection in a hypothetical standard mouse.

The curves obtained from the experiment with the Moscow strain of virus will be discussed in some detail. The Hampstead strain gave essentially similar results, but the virus concentrations were always somewhat lower.

### *The progress of infection through various organs*

Virus was first detected in the inoculated foot, then on the 4th day in the spleen, on the 4th and 5th day in the blood, and on the 6th day in the skin. As these data were not sufficiently detailed to enable a clear picture to be drawn of the pathogenesis of the natural infection, another experiment was carried out in which a number of mice were infected with the Moscow strain by rubbing a cotton-wool swab soaked with a concentrated suspension of virus on the right pinna. At intervals of hours and then of days after infection two mice were killed and the pinna, the draining cervical lymph node, the liver, spleen, and blood were tested for virus by the intraperitoneal inoculation of pooled tissue suspensions into mice. No attempt was made to titrate the virus content of the suspensions, but the method adopted, consisting of the inoculation into each of two mice of half the 20 per cent. suspension obtained by thorough grinding of the organ, ensured that even a very few virus particles would be detected. The intervals till death gave a rough measure of the amount of virus inoculated, and weight was given to them in constructing table IV, which shows the results of this experiment.

TABLE IV

*Occurrence of virus in several organs at intervals after infection by the application of Moscow ectromelia virus to the ear*

Material	Hours after infection				Days after infection				
	1	2	4	8	1	2	3	4	5
Pinna . . .	+	+	+	+	++	+++	+++	+++	++++
Regional lymph node	—	—	—	+	+	++	+++	+++	++++
Blood . . .	—	—	—	—	—	—	+	++	++
Liver . . .	...	...	...	...	—	—	++	+++	++++
Spleen . . .	...	...	...	...	—	—	++	+++	++++

### *Virus multiplication in the foot*

Between the 1st and 8th day after infection the virus content of the foot increased logarithmically. After the 8th day the curve

## MOUSE-POX (INFECTIOUS ECTROMELIA)



FIG. 5.—Section of skin of abdominal wall of a mouse 7 days after infection in the foot with Moscow ectromelia virus. The earliest lesion of the rash of mouse-pox. Localised area of epidermis showing slight proliferation, oedema and vacuolation of the epidermal cells. A few cytoplasmic inclusion bodies could be seen in sections stained with Mann's stain. The dermis is normal. Hæmatoxylin and eosin.  $\times 425$ .

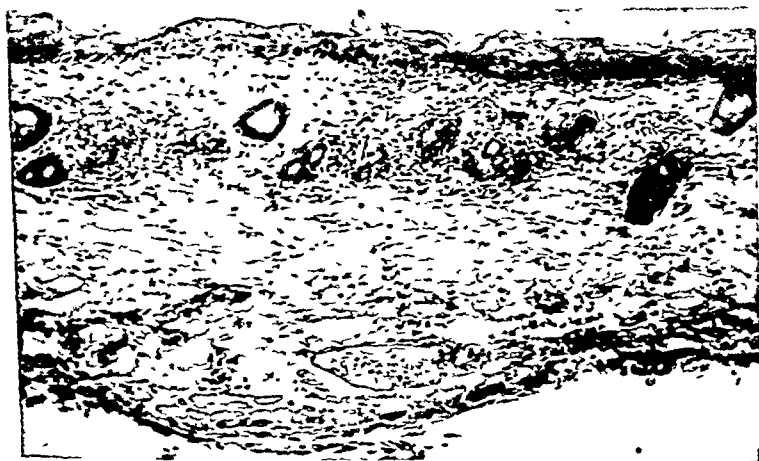


FIG. 6.—Section of skin of abdominal wall of a mouse 9 days after infection in the foot with Moscow ectromelia virus. Necrosis of epidermal cells, especially the superficial layer, and of cells of hair follicles is evident. There are many nuclear fragments and a few lymphocytes in the dermis, especially in the region of the necrotic hair follicles. Hæmatoxylin and eosin.  $\times 115$ .



flattened out and the concentration of virus remained approximately constant between the 9th and 14th day, although there were considerable individual variations in the different mice tested. After the 14th day, when the antibody titre reached its peak, the virus concentration fell steadily at about the same rate as it had risen. In most mice virus was not detected in the foot from the 24th day onwards, but occasional results were positive until the 31st day.

The inoculated feet remained normal in appearance until the 7th day, when there was a very slight degree of swelling. This point, which constituted the first clinical evidence of infection with mouse-pox, was taken as the end of the incubation period, and it corresponded to a very high concentration of virus in the foot. Œdema increased to reach a maximum at the 10th day. Ulceration then occurred, followed by scabbing and secondary bacterial infection, and the foot healed gradually until the scabs finally fell off about the 24th day.

With the Hampstead strain the form of the curve was similar, but the maximum titre was less and the foot lesions, which were less severe, healed more rapidly.

#### *Virus multiplication in the spleen and viræmia*

On the 4th day after infection, virus was demonstrated in the spleen and in very small amount in the blood. A period of logarithmic multiplication followed, and in certain animals the concentration of virus in the spleen reached the very high level of  $10^{10}$  infective particles per ml. Such high values were attained only in moribund animals, or in cases in which the severity of the lesions of the liver and spleen indicated that, although apparently healthy when killed, the mice would shortly have died. In the other animals a stationary phase was reached at a level of  $10^8$  infective particles per ml. on the 7th day and this was maintained until the 10th day. Thereafter, coinciding with the rapid increase in circulating antibody, the virus concentration in the spleen declined rapidly and none was found after the 16th day.

Large amounts of virus in the blood were found only in moribund animals. In all cases the virus concentration in the blood corresponded fairly closely, at a much lower level, with the virus concentration in the spleen, presumably because viræmia is due to continual liberation of virus from infected cells of the spleen and liver, and probably the bone marrow also, by necrosis of cells lining the sinusoids of these organs. Virus rapidly and permanently disappeared from the circulation when the antibody titre reached its peak. One thin sick mouse without clinical evidence of healing of the skin lesions was killed on the 16th day; it showed a high concentration of virus in the foot and skin, and virus was still present in the spleen and blood. The dilution phenomenon was evident with the blood, for the "undiluted" material (actual dilution 1 in 2) gave a negative result

and the blood diluted 1 in 200 caused infection of the inoculated mice. The E-AHA titre of the serum in this animal was 1000.

No macroscopic change was evident in the spleen until the 6th day, when the organ appeared engorged. Next day the spleen was completely necrotic in the two moribund animals examined, and varying degrees of change from a few foci to complete necrosis were seen each day until the 13th. Thereafter the spleen was usually pale in colour and any necrotic foci were gradually replaced by fibrous tissue. After the 18th day the spleen appeared normal, or there were patches of fibrosis and small raised plaques scattered over it.

With the Hampstead strain the curves for virus concentrations in the spleen and blood followed similar courses, but rose less rapidly and to a lower level ( $10^6$  infective particles per ml.) than with the Moscow strain. In two moribund animals killed on the 9th day, however, the virus content of the spleen and blood was almost the same as in a moribund animal killed on the 9th day of the experiment with the Moscow strain. Macroscopic lesions of the spleen were much less severe than with the Moscow strain, except for the two moribund animals just mentioned.

#### *Virus multiplication in the skin*

The curve showing the virus content of the skin and its relation to visible lesions of the skin and to the occurrence of inclusion bodies in the epidermal cells is of considerable interest. Virus was first demonstrated in the skin on the 6th day. It then increased rapidly in a logarithmic manner until the 8th day. From the 9th until the 13th day the virus concentration remained stationary at about the same level as had been attained in the spleen, and then fell steadily until, from the 18th day onwards, it could not be detected.

As in the case of the primary lesion of the foot and necrosis in the spleen, macroscopic changes in the skin were not seen until the virus concentration had reached its maximum level, on the 9th day of the experiment. The clinical course of the rash in this experiment was described in an earlier section. The relationship between virus content, macroscopic appearance and the number of inclusion bodies in the epidermal cells can be appreciated by a study of figs. 3 and 4. Fig. 8 shows widespread infection of the epidermis without visible lesions of the skin.

As with the other organs, the curve for the virus concentration in the skin in the experiment with the Hampstead strain followed the same course as in the Moscow strain experiment, but the maximum was lower. The skin lesions were less numerous, less extensive and less severe, they healed more rapidly and inclusion bodies were much less numerous in animals inoculated with the Hampstead strain.

The occurrence of many papules which increased in number for two or three days and the widespread but focal distribution of the

## MOUSE-POX (INFECTIOUS ECTROMELIA)



FIG. 7.—Section of skin of abdominal wall of a mouse 14 days after infection of the foot with Moscow ectromelia virus. The dermis is thickened and densely infiltrated with lymphocytes. No hair follicles can be seen. A thick scab is adherent to the necrotic epidermis on the left and on the right extend over regenerating epidermis. Hæmatoxylin and eosin.  $\times 115$ .



FIG. 8.—Section of skin of abdominal wall of a mouse which died 7 days after the inoculation of a large dose of Moscow ectromelia virus in the foot. Macroscopically the skin appeared normal except for a few small macules. Almost every cell in the epidermis and the hair follicle in the section illustrated contains one or more inclusion bodies. The dermis appears normal. Mann's stain.  $\times 220$ .



lesions indicate that infection of the epidermal cells must have resulted from a massive dissemination of virus through the blood stream. It is most unlikely that the primary viraemia which resulted in infection of the spleen caused this, for the amount of virus would be too small. The time relations of the curves indicate that the infection of the skin was due to the secondary viraemia associated with cellular necrosis in the liver and spleen.

#### *The development of serum antibody*

No antihæmagglutinin was detected until the 7th day. It then rose logarithmically to reach its peak on the 14th day, and declined slowly thereafter. The response with the Hampstead strain of virus was similar.

#### THE DEVELOPMENT OF SKIN HYPERSENSITIVITY TO ECTROMELIA VIRUS

It is evident from what has been said that the rash of mouse-pox is due to multiplication of virus in the epidermal cells and their subsequent necrosis. Many suggestions have been made that allergy plays some part in the development of the rash of the acute exanthemata, ranging from the view that the rash of the acute exanthemata (e.g. measles) is the expression of an allergic response to the virus (von Pirquet, 1913), to Burnet's (1940) suggestion that the points of weakness in the skin and mucous membranes which permit multiplication of the virus in these sites are due to a primarily allergic reaction there.

An experiment was carried out to determine the time of appearance of an allergic response to ectromelia virus. Groups of three or four mice were inoculated in the left fore foot with 500 infective particles of a saline suspension of Hampstead ectromelia virus prepared from infected egg membranes. At daily intervals from the 4th to the 14th day 0.05 ml. of a saline suspension of mouse liver containing about  $10^8$  infective particles of Moscow ectromelia virus was inoculated in the pad of the right hind foot and 0.05 ml. of a saline suspension of normal mouse liver in the left hind foot. The feet were examined daily and the degree of enlargement noted. The fore feet inoculated with Hampstead virus became swollen after the usual incubation period of 7 or 8 days. At most there was slight swelling on the first day after the inoculation of the normal mouse liver in the left hind foot. The progress of enlargement of the right hind foot, which had been inoculated with the concentrated suspension of Moscow ectromelia virus, is shown in fig. 9. Various intermediate groups are omitted from the figure for the sake of simplicity.

Only one of these mice died, although all non-immunised controls died within six days of inoculation with the concentrated suspension of Moscow virus. The fatal case occurred in the first group, and the animal died after several days' sickness on the 13th day, i.e. nine



days after the inoculation of the Moscow virus. Some degree of general immunity was therefore conferred by the inoculation of a small dose of Hampstead virus four days before the test inoculation. However, there was no evidence of local immunity, that is to say any alteration in the response of the inoculated feet to the test infection, until the interval between inoculations was six days. In this group the swelling progressed normally until the 4th day and then regressed. There was local evidence of immunity, therefore, on the 10th day.

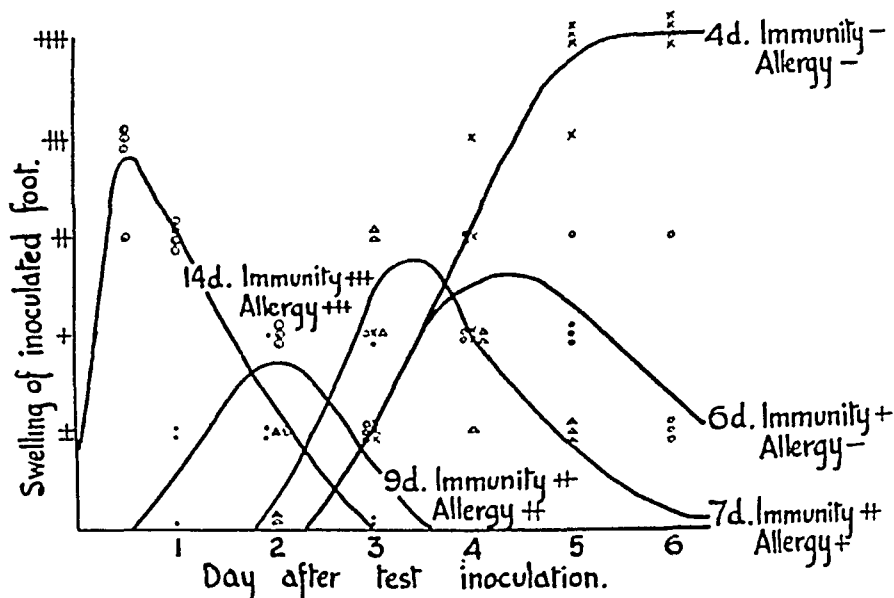


FIG. 9.—The development of immunity and allergy in mouse-pox is shown by the progress of enlargement of the hind foot inoculated with concentrated Moscow ectromelia virus at intervals of 4, 6, 7, 9 and 14 days after infection in another foot with a small dose of the Hampstead strain of the same virus. The 4-day curve represents the reaction of normal mice and the 14-day curve the reaction of completely immune animals.

The development of allergy was first seen in the seven-day group, in which a slightly accelerated response occurred on the 3rd day after the inoculation of the test dose of virus, that is ten days after the initial infection. Both immunity and allergy rapidly increased and 14 days after primary infection with the Hampstead strain there was a brisk allergic reaction which reached its peak within 12 hours, the foot regaining its normal size by the 3rd day.

Skin hypersensitivity thus appeared about the same time as local immunity, and at a time when the E-AHA titre was increasing rapidly, *i.e.* about ten days after infection. It seems unlikely, therefore, that allergy plays any part either in localising virus in the skin, as generalised infection of the skin occurs on the 6th day, or in the development of the rash.

## ROUTE OF INOCULATION AND TIME OF APPEARANCE OF THE RASH

The experiments described earlier showed that a period of 2-3 days elapses between natural infection or pad inoculation and the primary viraemia which initiates infection of the liver and spleen. If a dose of virus is inoculated intravenously this period of delay should be eliminated—that is, from inoculation the disease should follow a course as if it had begun at about day 3 on figs. 3 and 4, with consequent earlier appearance of the rash.

This was tested by inoculating groups of mice intravenously and in the pad of the hind foot with 0.05 ml. of suspensions containing 500 and  $5 \times 10^6$  infective particles of Hampstead ectromelia virus, and examining them daily to determine when the rash appeared. The results (table V) are exactly as predicted. The incubation period

TABLE V

*Effect of dosage and route of inoculation of Hampstead ectromelia virus on the times of appearance of the primary lesion and secondary rash*

Dose (no. of infective particles)	Route of inoculation	Time of appearance (days) of	
		primary lesion	secondary rash
500 . . . {	pad	$8.7 \pm 0.8$	$11.5 \pm 0.2$
	intravenous	...	$9.0 \pm 0.5$
$5 \times 10^6$ . . . {	pad	$5.7 \pm 0.5$	$9.2 \pm 0.7$
	intravenous	...	$7.0 \pm 1.0$

depended on the dose, but in both groups the animals inoculated intravenously developed a rash 2-3 days before those inoculated in the foot.

## Interpretation of these findings

The results of these experiments can be integrated and a hypothesis developed of the pathogenesis of mouse-pox, the essential features of which are shown in fig. 10.

Natural infection is initiated by the entrance of a few virus particles into the skin of the mouse through an abrasion, which may be minute. This effect is closely simulated by the inoculation of a small dose of virus into the pad of the foot. As McMaster and Hudack (1935) pointed out, intradermal inocula at once enter the lymphatics. Both in the natural disease and after pad inoculation, virus is carried by the lymphatics to the regional lymph nodes, where it can be demonstrated within eight hours of infection. After an initial lag phase, not investigated in these experiments, virus multiplication proceeds in a logarithmic fashion in the primary lesion and in the draining lymph node. When the concentration of virus in any organ or tissue

reaches a level of about  $10^7$  infective particles per g., macroscopic signs of infection appear in that organ. This occurs on the 7th day in the primary lesion and the swelling that results constitutes the first clinical evidence of infection, marking the end of the incubation period. Ulceration of the primary lesion occurs soon after and the mouse becomes infectious for the first time.

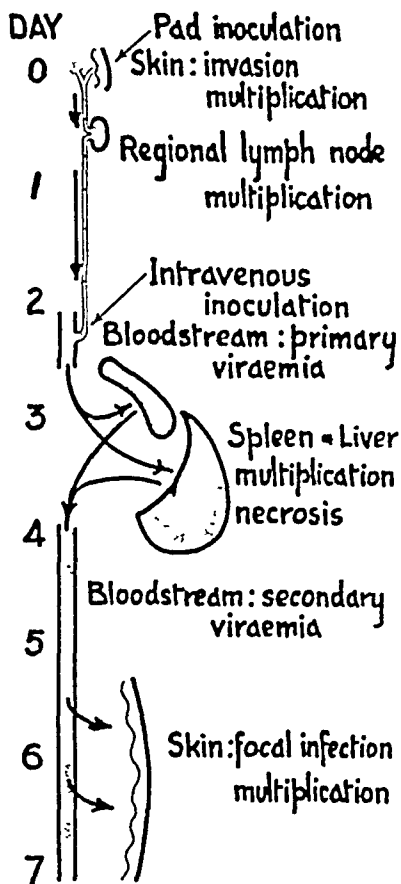


FIG. 10.—The pathogenesis of mouse-pox: a diagram illustrating the progress of infection during the incubation period.

During this relatively long incubation period there is an orderly sequence of multiplication and dissemination of virus. Multiplication of virus in the regional lymph node and the constant addition of virus from the primary lesion results in necrosis of the cells of the lymph node and the passage of virus through the efferent lymphatics to the blood stream. It is usually impossible to isolate virus from the blood stream at this stage, for the few particles which enter it are probably taken up rapidly by the macrophages of the liver and spleen, and possibly to a slight extent by those of the bone marrow also. Virus can be isolated from the liver and spleen on the 3rd day and it multiplies rapidly in both organs. Necrosis of infected cells lining the sinusoids liberates virus directly into the blood stream. By the 4th or 5th day this release of virus exceeds the capacity of the cells of the reticulo-endothelial system to dispose of it and the period of viraemia is established which lasts until the 13th day.

Virus distributed in this way is localised in several situations; almost invariably in the basal epidermal cells of the skin, commonly in the cells of the conjunctiva and near the lymph follicles of the intestine, rarely in the kidneys, lungs, submaxillary glands and pancreas. In the skin the virus multiplies rapidly in the cells of the epidermis, hair follicles and sweat glands, and when it reaches the critical level of about  $10^7$  infective particles per g. on the ninth day, pale flat papules appear which progress to ulceration in a few days. Several crops of skin lesions appear and merge into one another. This period, characterised by the liberation of large amounts of virus into the

environment, is the most infectious stage of the disease (Fenner, 1947b).

Intravenous inoculation of the virus eliminates the initial stages of invasion via the lymphatics, and with the same dose of virus the infection develops and the rash appears two or three days earlier after intravenous inoculation than after pad inoculation.

In acutely fatal cases of mouse-pox the concentration of virus in the liver and spleen reaches the very high level of  $10^{10}$  infective particles per g. The massive necrosis of infected cells of the liver and spleen causes death, which usually occurs on the 7th, 8th or 9th day with the doses under discussion, that is, too early for the rash to have become evident. This explains the observation noted in table II that in most acutely fatal cases a rash was not detected. Titration of the skin of these fatal cases and examination of suitably stained sections (*e.g.* fig. 8) show that the virus content of the skin may be very high.

Circulating antibody, presumably including other types of antibody as well as the antihæmagglutinin used as an index, first appears on the 7th day and increases logarithmically between the 7th and the 12th day to reach a maximum by the 14th day. It prevents the infection of new cells, the virus content of all tissues and organs falls rapidly and the virus can no longer be demonstrated in the internal organs or skin after the 18th day and in the foot after the 30th day.

## DISCUSSION

### *The growth curves of ectromelia virus in the mouse*

The growth curves of animal viruses have been investigated for various viruses and rickettsiæ grown in the chick embryo, namely influenza virus (Burnet, 1941; McLean *et al.*, 1944; Sigurdsson, 1944; Henle *et al.*, 1947); vesicular stomatitis virus (Sigurdsson, 1943a); equine encephalomyelitis virus (Bang, 1943); rabies virus (Sigurdsson, 1943b); and in mice, namely influenza (Smorodintseff and Ostrovskaya, 1937; Taylor, 1941); pneumonia virus of mice (Curnen and Horsfall, 1946); murine typhus rickettsia in the mouse (Ipsen, 1945).

The most precise studies are those made recently by Henle *et al.*, who adapted the one-step growth curve technique used for bacterial viruses to influenza virus infection of the allantoic membrane. They found a lag period of 6-9 hours during which multiplication of the virus occurred, and then a period of two hours during which the newly formed virus was released from the infected cells. If no measures were taken to prevent further infection of cells the "steps" quickly merged and the final curve was then similar to that obtained by other workers on the growth of viruses in the chick embryo; *i.e.* after an initial lag phase there was a period during which the virus content increased logarithmically.

The curves for virus increase in figs. 3 and 4 all show a logarithmic phase, and table VI shows the generation times (*i.e.* the times required

TABLE VI

*The generation times \* of ectromelia virus in different tissues*

Organ or tissue	Period of test (days after inoculation)	Generation time (hours)	
		Mo-cow strain of virus	Hampstead strain of virus
Foot . . .	2.8	0.4	10.1
Skin . . .	6.8	2.4	2.6
Spleen . . .	4.7	2.9	6.8
	4.9 (including fatal cases)	...	5.0

\* The generation time is the time required for a two-fold increase in virus concentration.

for a two-fold increase in the virus concentration) for the two strains of ectromelia virus in the various tissues during this logarithmic phase. The slopes of the curves were determined by the method of least squares applied to all data available for the period indicated on the table. In table VII the generation times have been worked out for

TABLE VII

*The generation times \* of various viruses and rickettsiae (after several authors)*

Virus or rickettsia	Host and conditions	Generation time	Author
Eastern equine encephalomyelitis	Chick embryo, 8 days old, 35-36° C.	30 minutes	Bang (1943)
Vesicular stomatitis .	Chick embryo, 7 days old, 35-36° C. and 39-40° C.	26 "	Sigurdsson (1943b)
	Chick embryo, 10 days old, 35-36° C.	40 "	"
Influenza B (LEE) .	Chick embryo, 11 days old, 35 and 37° C.	68 "	McLean <i>et al.</i> (1944)
Rabies . . . .	Chick embryo, 8 days old, 35-36° C.	9 hours	Sigurdsson (1943b)
<i>Rickettsia mooseri</i> . .	Mouse liver, intraperitoneal inoculation	2.5 "	Ipsen (1945)
Pneumonia virus of mice (PVM)	Mouse lung, intranasal inoculation	6 "	Curnen and Horsfall (1946)
Influenza . . . .	Mouse lung, intranasal inoculation	2.4 "	Taylor (1941)

\* The generation time is the time required for a two-fold increase in virus concentration

a number of viruses from graphs given by the authors quoted. In all cases the generation times were estimated from the logarithmic phase of the graphs. In calling the time required for a two-fold increase in virus concentration the "generation time" of the virus a number of complicating factors have been ignored. The method of estimation depends upon the number of infective particles which

can be detected in certain organs of a number of different eggs or mice at various intervals after infection. Variable factors which have been ignored include: (a) the occurrence of non-infective virus particles, (b) aggregation of virus particles, (c) failure to release all virus particles from infected cells, (d) possible repeated addition of virus to the tissue under study (*e.g.* repeated infection of the mouse skin by virus circulating in the blood), and (e) individual variations in the resistance of the eggs and mice used as the source of the virus. However, the figures determined indicate the order of the speed of reproduction of the viruses, and when comparisons are made between the generation times of different strains of the same virus in the same organ (*e.g.* the mouse spleen), many of the variables mentioned above are eliminated.

It will be seen that the generation times of ectromelia virus in the spleen and in the skin are of the same order as those found for other viruses and rickettsias in the mouse. The rate of increase of virus in the foot is considerably less, and this is of considerable significance in relation to the time of appearance of the primary lesion. That the rate of increase of virus in primary lesions acquired by contact is much the same as in the inoculated foot is indicated by the similar incubation periods and the finding that in all organs and tissues macroscopic change is evident when the virus content reaches about  $10^7$  infective particles per ml.

The generation times of the Moscow ectromelia virus were uniformly less than those of the Hampstead strain, but the difference was pronounced only in the spleen. It is likely that the relative rates of growth of the two strains in the liver are parallel to those found for the spleen, to judge from hæmagglutinin titrations and the macroscopic appearance of the liver at different stages. This is probably of considerable significance in deciding the outcome of infection with the two strains. With both, the liver and spleen are infected at the same time, on the 3rd or 4th day. The more rapid multiplication of the Moscow strain in the liver and spleen results in high virus concentrations being attained in these organs before antibody production begins, and the resulting massive necrosis of the infected cells results in death of the infected mice.

Although the generation times of the two strains in the skin are the same, the skin lesions are much more severe with the Moscow strain. This is probably due to the much more widespread focal infection of the skin in consequence of the much higher viræmia characteristic of that strain. This in its turn is a consequence of the higher virus concentrations in the liver and spleen associated with the Moscow strain.

On the 8th or 9th day a new influence exerts a decisive effect upon the course of the disease. If this were not so it would be a matter of only a few days before the concentrations attained by the Hampstead strain reached the same high levels as those reached by the Moscow strain, with corresponding slightly delayed but equally severe lesions

and similar death-rate. The new influence could not be the exhaustion of susceptible cells, for both strains reach very high concentrations in the spleen and liver of acutely fatal cases. Antibody was first detected about this time and its titre increased logarithmically between the 8th and the 14th day, that is, the stationary phase of virus multiplication. But logarithmic multiplication of the virus did not continue to the point where interference with the function of the liver and other organs caused death from necrosis of their cells—that is, logarithmic multiplication ceased before the concentration of virus in the spleen reached  $10^{10}$  infective particles per g., and the cessation was probably due to the increasingly effective action of antibody in preventing the infection of additional cells. At the end of the stationary phase most of the virus in the host was shed, either externally in the discharge from skin lesions or internally by necrosis of the cells of the infected organs. As further infection of cells was now prevented by antibody, the concentration fell rapidly until the virus was eliminated completely.

That this is not the complete or only explanation of a virus growth curve consisting of a phase of logarithmic multiplication, a stationary phase and a phase of logarithmic decline in concentration is indicated by Burnet's (1941) experiment on influenza-virus infection in the allantoic cavity of the chick embryo, in which he obtained such a curve but was unable to demonstrate any antibody in hatched chicks infected 9 and 14 days earlier. In this case the exhaustion of susceptible cells and the decreased sensitivity of older cells to virus infection were probably of importance in determining the shape of the virus growth curve.

In light infections of the chorio-allantois with ectromelia virus Burnet and Lush found that a layer of uninfected ectoderm formed beneath the initial lesion and that the cells of this layer, although in contact with infected and necrotic cells, did not become infected but underwent partial keratinisation, the infected and necrotic material being cast off. This is not unlike the process of healing of the skin lesions of mouse-pox, and in the egg it is presumably accomplished without the intervention of antibody.

It might be objected that the decline in the concentration of virus after the 14th day is more apparent than real and may be accounted for by inactivation of virus by antibody during the titrations. Two observations show that this is not so. First, histological examination of the skin showed a rapidly diminishing number of inclusion bodies between the 14th and the 18th day, and the newly formed epithelial cells did not become infected. Second, the inactivating effect of antibody is negligible when it is diluted more than 100-500 times (Burnet, Keogh and Lush, 1937) and all titrations which gave figures of 5 log. units or more were diluted at least 500 times. With undiluted immune sera the apparent reduction in the concentration of suspensions of ectromelia virus titrated on the chorio-allantois or by the intra-

peritoneal inoculation of mice was to between 1 and 10 per cent. of the true figure (Burnet and Lush). As further evidence of the relative unimportance of virus inactivation in affecting the results of titrations, the dilution phenomenon was observed only once—with blood (E-AHA titre 1000) tested in mice on the 16th day of the experiment.

The only other investigations in which the stationary phase and phase of decline in concentration were discussed are those of Taylor on influenza and Curnen and Horsfall on pneumonia virus of mice. Both found that a striking fall occurred between the 7th and 10th day (Taylor) or the 8th and 12th day (Curnen and Horsfall). Taylor considered that the fall in concentration was probably due to the effect of antibody in preventing liberated virus from infecting additional cells. Curnen and Horsfall were unable to find antibody or masked virus when they tested the lungs of mice up to 12 days after infection, and they thought that the decline could not be explained exclusively on the basis of neutralisation by antibody produced in response to the infection. Ipsen's curves for rickettsiæ in the liver of infected mice show that in animals inoculated with a non-lethal dose the concentration begins to fall about the 8th day. With vaccinated mice inoculated intraperitoneally the fall began on the 4th or 5th day, mostly before the rickettsial content of the liver had reached a lethal concentration. He did not titrate the antibody in the organs or serum.

Further light may be cast on the importance of antibody in controlling infection in mouse-pox by quantitative studies of the virus content of various organs in groups of mice which have been actively or passively immunised, and experiments on these lines are now being performed.

### *The pathogenesis of mouse-pox*

The investigations described in this paper were undertaken in order to gain some insight into the reason for the long incubation period of the acute exanthemata of man and the pathogenesis of the rash in these diseases, mouse-pox being regarded as a satisfactory laboratory model for such purposes. The application of the hypothesis elaborated here for the pathogenesis of mouse-pox to the acute exanthemata of man is discussed elsewhere (Fenner, 1948b). The present discussion will be restricted to a comparison of the results of the experiments described in this paper with those of other investigations into the pathogenesis of generalised bacterial and virus diseases.

The classic studies on the mechanism of infection in bacterial diseases are those made by Ørskov and his collaborators (for reviews see Ørskov, 1932; Madsen, 1937). In mouse typhoid Jensen (1929) found that after ingestion of a small infective dose of bacilli invasion



of the regional lymph nodes occurred within 24 hours of administration. The next organs affected were the liver and spleen, in which organisms were detected in some animals on the 3rd day and in all by the 4th day after infection. He was unable to isolate bacteria from the blood until the 4th day, when bacterial multiplication in the liver and spleen "overflowed" into the circulation, although infection of the liver and spleen must have occurred via the blood stream. Further focal localisation of organisms sometimes followed in peripheral lymph nodes and elsewhere. The sequence of events in mouse-pox is very similar to this, the principal difference being that in mouse-pox virus multiplies locally at the site of entry into the host and causes a local primary lesion. This difference is probably due to the affinity of ectromelia virus for epidermal cells, which is also responsible for its later localisation there to cause the rash.

In experiments with vaccinia virus inoculated intradermally in young rabbits, Ørskov and Andersen (1938) found that the sequence of events was almost exactly the same as we have found for ectromelia virus. With very young animals the primary viraemia and invasion of the liver and spleen occurred very early and in animals which survived long enough, late secondary generalisation occurred, resulting sometimes in focal infection of the kidneys and skin. As a primary lesion occurred at the site of the intradermal inoculation the analogy with mouse-pox is complete, as might be expected from the close relationship of the two viruses. However, the generation time of vaccinia virus in rabbit skin must be higher than that of ectromelia virus in the mouse foot, or else very large doses were used, for the incubation period was much shorter.

The comparatively long incubation period of mouse-pox may be explained by two observations. First, the generation time of the virus in the primary lesion is long (10 hours) and macroscopic signs of infection do not appear until the virus concentration reaches a critical level of about  $10^7$  infective particles per g.; therefore the primary lesion does not appear for several days. Second, during the incubation period there is a step-by-step infection of several organs, which does not result in infection of the skin until the 5th or 6th day. Although the generation times of virus deposited in the skin and spleen are short, the virus concentrations cannot usually reach the critical level of  $10^7$  infective particles per g. until some days after the virus has reached this level in the primary lesion. In generalised diseases in which the generation time of the causative organism at its site of entry into the host is short, the local primary lesion may precede the symptoms of generalised infection by as much as a week, *e.g.* in rickettsial-pox (Greenberg *et al.*, 1947). In infections like influenza, on the other hand, symptoms are due to multiplication of the virus at its site of lodgement in the respiratory tract, and in this site the virus has a short generation time. The incubation periods of such diseases are therefore short.

## SUMMARY

The clinical features of naturally acquired mouse-pox are the primary lesion and the secondary rash. The development and appearance of these lesions and their histological features are described.

Growth curves were determined for two strains of ectromelia virus by daily titrations of virus in the inoculated foot and in the spleen, blood and skin of mice infected with small doses of virus, and detailed qualitative studies were made of the times of appearance of virus in the regional lymph node, spleen and liver, and blood of infected mice. The results showed that ectromelia virus underwent a step-by-step invasion of the body via the regional lymph node to the liver and spleen, which were reached on the third day. Multiplication of virus in these organs caused a secondary viræmia, during which multiple foci of infection of the skin resulted in the development of the rash some days later. Signs and symptoms of infection never occurred before the virus concentration in the tissue or organ reached a level of about  $10^7$  infective particles per g.

The generation times of the two strains of virus in different tissues were determined from the growth curves. The late appearance of the primary lesion was due to the slow multiplication of virus in the skin. The generation times of the two strains of virus were much the same except in the spleen, in which the Moscow strain multiplied more than twice as fast as the Hampstead strain. This difference, which probably occurs in the liver also, is directly responsible for the higher virulence of the Moscow strain and indirectly for the greater severity of skin lesions in the survivors.

This study of mouse-pox has important implications in the field of the acute exanthemata of man.

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# A SELECTIVE MEDIUM FOR ISOLATING STAPHYLOCOCCUS BASED ON THE DIFFERENTIAL INHIBITING EFFECT OF INCREASED CONCENTRATIONS OF SODIUM CHLORIDE

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HILL and White (1929) suggested the use of media with high concentrations of sodium chloride for inhibiting Gram-negative bacilli and the isolation of Gram-positive cocci. They found that such media markedly inhibited the growth of bacilli of the typhoid-paratyphoid, dysentery, diphtheroid and colon groups, of species of *Proteus* and *Pseudomonas* and of *Bacillus anthracis*. The Gram-positive cocci which they studied tolerated high salt concentrations and were viable on transfer from 20 per cent. sodium chloride agar. In sodium chloride broth they found the same differential bacteriostasis but to a lesser degree. Later Koch (1942) described a nutrient agar medium containing 7.5 per cent. NaCl for the selective isolation of staphylococci. This work was confirmed by Chapman (1945), who employed a 7.5 per cent. NaCl agar medium for isolating staphylococci from faeces and found it superior to other methods of isolation.

Recently Oddy and Clegg (1947), investigating staphylococcal food poisoning originating from pressed pickled beef, stated that their observations agreed with those of Kelly and Dack (1936), who noted that staphylococci would grow in salted meat with a content of 10 per cent. NaCl, which prevented the increase of sporing and non-sporing bacilli. Arising from this, Clegg and Oddy (personal communication, 1947) found that nutrient broth and Robertson's meat medium containing 10 per cent. added NaCl were selective for staphylococci and that the meat medium was better than the broth.

This paper reports experiments which were made to measure the inhibitory effect of NaCl in media and to assess the value of such media for the selective cultivation of staphylococci. They also served as a basis for investigating the mechanism of the inhibitory effect of NaCl.

## TECHNICAL METHODS

The amount of NaCl in media is expressed throughout as the amount of added NaCl. When the term "salt" is used to describe a medium it indicates that 10 per cent. NaCl had been added.

One strain of *Staphylococcus pyogenes* was used unless otherwise stated. It was coagulase-positive and produced a golden pigment. For inoculation, all strains of staphylococci were grown aerobically for 18 hours at 37° C. in nutrient broth. The culture was rapidly shaken in a mechanical shaker for 2-3 minutes to break up clumps. Stained films showed that about 70 per cent. of the cells were single, that the remainder were nearly all in pairs and that only a few were in groups of more than two. Thus it was possible to have a

reasonably uniform inoculum in successive experiments. A series of ten-fold dilutions of the culture was made in distilled water immediately before use. Since the amount of inoculum was always one drop (0.02 c.c.) per tube from a calibrated dropping pipette, its bacterial content is stated in terms of dilution of culture. The amount of medium was 5 c.c. unless otherwise stated.

To counteract the effect of chance distribution of organisms in the higher dilutions five tubes were always inoculated with each dilution tested.

## EXPERIMENTAL RESULTS

### I. *Growth of a strain of Staph. pyogenes in nutrient broth with increasing concentrations of NaCl*

Concentrations of NaCl ranging from 1 to 20 per cent. by intervals of 1 per cent. were inoculated with a series of dilutions of culture from  $10^{-1}$  to  $10^{-9}$ . Counts made by the method of Miles and Misra (1938) showed that the number of organisms in the undiluted inoculum was about  $2000 \times 10^6$ , so that one drop of  $10^{-7}$  dilution would contain about 4 organisms.

The results are shown in tables I, II and III. Each table represents a separate experiment with its own control but as controls were

TABLE I

*Growth of Staph. pyogenes in broth with increasing amounts of NaCl from 6 to 10 per cent.*

Dilution of inoculum	Control nutrient broth		Amount of NaCl added									
			6 per cent.		7 per cent.		8 per cent.		9 per cent.		10 per cent.	
	Incubation (hours)		Incubation (hours)		Incubation (hours)		Incubation (hours)		Incubation (hours)		Incubation (hours)	
	24	48	24	48	24	48	24	48	24	48	24	48
$10^{-4}$	5	5	5	5	5	5	5	5	5	5	5	5
$10^{-5}$	5	5	5	5	5	5	5	5	5	5	5	5
$10^{-6}$	5	5	5	5	5s	5	5s	5	0	5	1	5
$10^{-7}$	5	5	4	5	2s	4	0	5	0	4	1	5
$10^{-8}$	1	1	0	1	0	1	1	1	0	1	0	2
$10^{-9}$	0	0	0	0	0	0	0	0	0	0	0	0

The figures indicate the number of 5 inoculated tubes which showed growth macroscopically. s = slight.

similar the experiments can be assessed as a whole. The results with concentrations of NaCl from 1 to 5 per cent. have not been tabulated as there was nothing significant beyond a slight delay in growth. Higher concentrations of NaCl prevented growth or delayed it, or diminished the total amount, depending upon the concentration of NaCl, size of inoculum and period of incubation. When growth was delayed it was diminished in amount, even on prolonged incubation. Thus similar cultures were obtained with large inocula in high concentrations and small inocula in low concentrations. Even with 20 per cent. NaCl there was not complete inhibition if the inoculum

was large enough. In concentrations of NaCl which prevented growth the staphylococci remained viable and could be subcultured, but the limits of this have not been determined.

The data governing our choice of a concentration of NaCl suitable for a selective medium were afforded by cultures inoculated with a

TABLE II

*Growth of Staph. pyogenes in broth with increasing amounts of NaCl from 11 to 15 per cent.*

Dilution of inoculum	Control nutrient broth		Amount of NaCl added									
			11 per cent.		12 per cent.		13 per cent.		14 per cent.		15 per cent.	
	Incubation (hours)		Incubation (hours)		Incubation (hours)		Incubation (hours)		Incubation (hours)		Incubation (hours)	
	24	48	24	48	24	48	24	48	24	48	24	48
10 <sup>-1</sup>	5	5	5	5	5	5	5	5	5s	5	5vs	5
10 <sup>-2</sup>	5	5	5	5	5	5	5	5	5s	5	0	5
10 <sup>-3</sup>	5	5	5s	5	0	5	0	5	0	5	0	5s
10 <sup>-4</sup>	5	5	5vs	5	0	5	0	5	0	5	0	5vs
10 <sup>-5</sup>	5	5	0	5	0	5	0	5	0	5	0	5vs
10 <sup>-6</sup>	5	5	0	5	0	5	0	4	0	3s	0	0
10 <sup>-7</sup>	2	2	0	2	0	2	0	2s	0	1s	0	0
10 <sup>-8</sup>	0	0	0	0	0	0	0	0	0	0	0	0

The figures indicate the number of 5 inoculated tubes which showed growth macroscopically. s = slight; vs = very slight.

TABLE III

*Growth of Staph. pyogenes in broth with increasing amounts of NaCl from 16 to 20 per cent.*

Dilution of inoculum	Control nutrient broth			Amount of NaCl added														
				16 per cent.			17 per cent.			18 per cent.			19 per cent.			20 per cent.		
	Incubation (hours)			Incubation (hours)			Incubation (hours)			Incubation (hours)			Incubation (hours)			Incubation (hours)		
	24	48	72	24	48	72	24	48	72	24	48	72	24	48	72	24	48	72
10 <sup>-1</sup>	5	5	5	5s	5s	5s	5s	5s	5s	0	0	5vs	0	0	5vs	0	0	5vs
10 <sup>-2</sup>	5	5	5	0	5s	5s	0	5s	5s	0	0	5vs	0	0	5vs	0	0	5vs
10 <sup>-3</sup>	5	5	5	0	5s	5s	0	5s	5s	0	0	5vs	0	0	5vs	0	0	0
10 <sup>-4</sup>	5	5	5	0	5s	5s	0	5s	5s	0	0	5vs	0	0	5vs	0	0	0
10 <sup>-5</sup>	5	5	5	0	5s	5s	0	0	5vs	0	0	5vs	0	0	0	0	0	0
10 <sup>-6</sup>	5	5	5	0	5s	5s	0	0	5vs	0	0	0	0	0	0	0	0	0
10 <sup>-7</sup>	5	5	5	0	0	5vs	0	0	0	0	0	0	0	0	0	0	0	0
10 <sup>-8</sup>	5	5	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
10 <sup>-9</sup>	2	2	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
10 <sup>-10</sup>	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

The figures indicate the number of 5 inoculated tubes which showed growth macroscopically. s = slight; vs = very slight.

10<sup>-7</sup> dilution, this inoculum by calculation containing less than 10 cocci. With concentrations of NaCl up to 12 per cent., subject to probable technical discrepancies, there was some delay but no failure of growth, but above 12 per cent. progressive failure of growth was evident. Therefore 10 per cent. was chosen as a suitable amount to be added to broth for selective isolation of staphylococcus.

## II. Effect of adding 10 per cent. NaCl to broth on the growth of other strains of staphylococci

Five further strains tested as described in section I included coagulase-positive *aureus* and *albus* and coagulase-negative *albus* strains. The minimal inoculum for broth was  $10^{-7}$  and with two strains it was the same for salt broth. Three strains required  $10^{-6}$  for salt broth, but even this was by calculation fewer than 100 organisms. After 24 hours there was less growth in salt broth than in broth but at 48 hours it was almost equal.

## III. The inhibitory effect of NaCl in broth on *Bacterium coli*, *Proteus vulgaris*, *Bacterium friedländeri* and *Salmonella typhi*

These organisms were investigated as described for staphylococci in section I. In table IV the concentrations of salt which inhibited

TABLE IV

Comparison of inhibitory effect of NaCl in broth on different organisms after incubation for 96 hours

Organism	No. of bacteria in the largest inoculum tested	Lowest percentage of added NaCl which inhibited the largest inoculum	No. of bacteria in the smallest inoculum tested	Lowest percentage of added NaCl which inhibited the smallest inoculum
<i>Staph. pyogenes</i> . . .	$4 \times 10^6$	>20	<10	15
<i>Bact. coli</i> . . .	$7 \times 10^6$	7	<10	4
<i>Proteus</i> . . .	$4 \times 10^6$	0	<10	7
<i>Bact. friedländeri</i> . . .	$4 \times 10^6$	0	<10	7
<i>Salm. typhi</i> . . .	$6 \times 10^6$	0	<10	3

large and small inocula are stated for these organisms in comparison with a staphylococcus. It will be seen that the staphylococcus was much more resistant than the others to NaCl and that 10 per cent. NaCl, although not inhibiting small inocula of the staphylococcus, completely inhibited large inocula of the other organisms.

Six other strains of *Bact. coli* were tested by inoculating an undiluted overnight broth culture into salt broth; none grew even after incubation for seven days.

Within the range of salt concentrations permitting growth, there was the same gradation of effect as with staphylococci. This ranged from complete suppression of growth to different degrees of delay and diminution, again depending on size of inoculum and NaCl concentration. In concentrations of salt which prevented growth there was survival for a time and it was possible on occasion for these organisms to appear in subculture.

#### IV. Tests made with known mixtures of bacteria in 10 per cent. salt broth

Salt broth was inoculated simultaneously with undiluted overnight broth cultures of *Staph. pyogenes*, *Bact. coli*, *Bact. friedländeri* and *Pr. vulgaris*. This was incubated for about 18 hours and subcultured to a blood-agar plate which overnight yielded an apparently pure culture of the staphylococcus. Although the strain of *Pr. vulgaris* used did not grow in the salt broth, it survived and spread over the plate after further incubation.

A series of similar cultures was made by inoculating progressive ten-fold dilutions of the staphylococcus along with all the other bacteria. In 24 hours, a broth control of staphylococcus alone grew down to  $10^{-7}$ ; in salt broth mixed with other bacteria to  $10^{-6}$ . After 48 hours there was growth in the salt broth with  $10^{-7}$  and a subculture on blood agar yielded overnight an apparently pure culture of *Staph. pyogenes* from which separate colonies could easily be picked for final isolation. Thus this medium readily demonstrated a small number of staphylococci, calculated as about 10, in an inoculum known to contain about 150 million other bacteria.

#### V. Addition of NaCl to other basal media

*Robertson's meat medium.* Clegg and Oddy (personal communication) thought that meat medium with salt was a better selective medium for staphylococci than salt broth. The growth of staphylococci was therefore tested in meat medium containing added amounts of NaCl from 2 to 20 per cent. The salt was added to broth which was poured on the meat before autoclaving, each tube containing approximately 2 g. of meat and 10 c.c. of broth.

Certain selected results are recorded in table V for comparison with tables I, II and III, to indicate the difference between salt meat and salt broth. Salt meat was less inhibitory and its chief advantage as a selective medium was that small inocula grew faster: compare, for example, inocula of  $10^{-6}$  and  $10^{-7}$  in 10 per cent. salt at 24 and 48 hours (tables I and V). It did not detect smaller numbers of staphylococci.

That the inhibitory effect of NaCl was less in meat medium than in broth was shown also by the findings that with similar inocula a larger amount of NaCl was required to delay growth in meat and that certain inocula grew in high concentrations of salt in meat but not in broth (tables I, II, III and V). In salt meat too growth was a little heavier.

*Sodium thioglycollate medium.* This was made from meat-infusion broth—the standard broth used throughout—by adding to it 0.1 per cent. sodium thioglycollate and 0.5 per cent. agar. In this medium staphylococci grew well in a zone at the surface, but NaCl was much more inhibitory in it than in broth or Robertson's meat. In thio-



glycollate broth an inoculum of  $10^{-8}$  grew well in 24 hours, but when 10 per cent. salt was added the smallest inoculum that grew in 24 hours was  $10^{-3}$ ; after 48 hours it was only  $10^{-6}$ .

TABLE V

*Growth of Staph. pyogenes in Robertson's meat medium with different amounts of added NaCl*

Dilution of Inoculum	Control. Robertson's meat medium			Amount of NaCl added														
				10 per cent.			14 per cent.			16 per cent.			18 per cent.			20 per cent.		
	Incubation (hours)			Incubation (hours)			Incubation (hours)			Incubation (hours)			Incubation (hours)			Incubation (hours)		
	24	48	72	24	48	72	24	48	72	24	48	72	24	48	72	24	48	72
$10^{-1}$	5	5	5	5	5	5	5	5	5	5	5	5	5 <sup>s</sup>	5 <sup>s</sup>	5 <sup>s</sup>	0	0	5 <sup>s</sup>
$10^{-2}$	5	5	5	5	5	5	5	5	5	5	5	5	0	0	5 <sup>s</sup>	0	0	5 <sup>s</sup>
$10^{-3}$	5	5	5	5	5	5	5	5	5	5 <sup>s</sup>	5 <sup>s</sup>	5	0	0	5 <sup>s</sup>	0	0	5 <sup>vs</sup>
$10^{-4}$	5	5	5	5	5	5	5	5	5	0	5 <sup>s</sup>	5	0	0	5 <sup>s</sup>	0	0	5 <sup>vs</sup>
$10^{-5}$	5	5	5	5	5	5	5 <sup>s</sup>	5 <sup>s</sup>	5	0	5 <sup>s</sup>	5 <sup>s</sup>	0	0	5 <sup>s</sup>	0	0	0
$10^{-6}$	5	5	5	5	5	5	3 <sup>s</sup>	5 <sup>s</sup>	5	0	5 <sup>vs</sup>	5 <sup>s</sup>	0	0	5 <sup>s</sup>	0	0	0
$10^{-7}$	5	5	5	5	5	5	0	5 <sup>s</sup>	5 <sup>s</sup>	0	0	5 <sup>s</sup>	0	0	5 <sup>s</sup>	0	0	0
$10^{-8}$	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

The figures indicate the number of 5 inoculated tubes which showed growth macroscopically. s = slight. vs = very slight.

*Digest broth.* This was made from a pancreatic digest of beef without peptone or salt (Mackie and McCartney, 1948). In this medium too the inhibitory effect of 10 per cent. NaCl on staphylococci was more marked than in nutrient broth or meat medium. There was a marked delay in growth and in the suppression of small inocula.

*Nutrient agar.* This contained 2.5 per cent. of agar in the basal nutrient broth. A comparison of agar with and without 10 per cent. NaCl was made by counting the number of colonies which grew from the standard inocula of staphylococci on the surface of plate cultures.

On plain agar a dilution of  $10^{-7}$  gave in 24 hours a mean of 4 colonies, equivalent to a viable count of  $2000 \times 10^6$  organisms per c.c. This inoculum always grew in broth and thus there was little difference between plain agar and broth. On salt agar there was no visible growth except with the largest inocula after 24 hours. At 48 hours the  $10^{-6}$  inoculum gave less than 10 colonies, equivalent to a viable count of less than  $500 \times 10^6$  organisms per c.c. In salt agar there was therefore more delay than in salt broth, and suppression of small inocula. The inferiority of salt agar might be ascribed to the physical properties of the gel.

#### VI. Tests made with 25 other strains of *Staphylococcus* in salt-meat medium

Fifteen coagulase-positive *aureus* strains and ten coagulase-negative *albus* strains, recently isolated directly on blood agar from

the nose, were examined to see whether there was general conformity among staphylococci regarding their ability to grow well in salt meat. All these strains behaved similarly and corresponded to that described in section V. A minimal inoculum of  $10^{-7}$ , calculated as less than 100 organisms, always grew; sometimes an inoculum of  $10^{-8}$  sufficed for growth, and there was no difference between salt-meat and ordinary-meat medium. After 18-24 hours a good culture was produced although growth was slightly less in salt meat.

#### VII. Tests with known mixtures of bacteria in salt-meat medium and its liquor

As a severe test of the selective quality of salt-meat medium for Staphylococcus a series of tenfold dilutions of this organism was inoculated along with undiluted cultures of *Salm. typhi*, *Salm. paratyphi* B, *Shigella sonnei*, *Shigella flexneri*, *Bact. coli*, *Str. pyogenes*, *Ps. pyocyanea* and *B. subtilis*. The cultures were incubated overnight and plated on blood agar. From the  $10^{-8}$  dilution of Staphylococcus, calculated as about 10 organisms, many colonies grew on the plate, from which a pure culture could readily be made. *B. subtilis* also came through in moderate numbers but did not obscure the staphylococci.

*Pr. vulgaris* was shown to be more resistant to salt than *Bact. coli* and *Salm. typhi* (table IV) and in a heavy mixed inoculum it may be found in subculture and may overgrow the plate. With a moderate inoculum incubated overnight (section IV) this did not happen. When it was added to the heavy mixed inoculum of these tests and incubated overnight Proteus appeared in subculture even if inoculated in a dilution of  $10^{-8}$ . In practice a pure culture of Staphylococcus can nevertheless be easily isolated by transferring a colony to salt meat and repeating the culture, when the small numbers of Proteus will be suppressed. Or subcultures may be made from the original salt meat to a medium inhibiting the spread of Proteus. It should be realised however that the mixed inoculum was intentionally made very heavy: in routine use inocula are such that difficulty from Proteus overgrowing the plate in this way has hardly ever arisen. The liquor from salt-meat medium gave results identical with those given by the medium itself (see section VIII).

In cultures from numerous samples of faeces Proteus seldom appeared when the original culture in salt meat was subcultured after overnight incubation. When it appeared, as it did in about 5 per cent. of cultures, it did not overgrow the Staphylococcus completely and a pure culture of staphylococci from a single colony could always be obtained. Small colonies of micrococci were sometimes present but caused no difficulty. The usual result was a pure culture of Staphylococcus on the plate, whether the inoculum was faeces, mouth washes or other material with a very mixed flora.

VIII. *Some factors affecting the inhibitory action of NaCl on the growth of Staphylococcus*

The mechanism by which NaCl inhibits the growth of bacteria is not known but a few points emerged during the study of *Staphylococcus* which are relevant to this problem.

*Peptone.* In section IV it was noted that digest broth grew staphylococci at least as well as nutrient broth, but that when 10 per cent. NaCl was added to each the digest broth was much more inhibitory, the minimal inocula producing cultures in 24 hours being  $10^{-2}$  in digest and  $10^{-8}$  in nutrient broth. This difference suggested that peptone, or the lack of it in digest broth, might be concerned. Therefore 0.5 c.c. of a solution containing 1 per cent. peptone and 0.5 per cent. NaCl was added to 5 c.c. of digest broth containing 10 per cent. NaCl. This medium then grew staphylococci as well as nutrient salt broth. Four brands of peptone (Difco; Witte; Evans, Sons, Lescher and Webb; and J. W. Towers & Co.) were tested more thoroughly. All were effective with only small quantitative differences. It was thus evident that peptone counteracted the inhibitory effect of NaCl.

It is not known what substances in peptone are responsible or how they act. A few preliminary tests indicated that serum and an alkaline extract of defibrinated blood behaved similarly.

*Extract of cooked meat.* The property of salt-meat medium which rendered it less inhibitory to *Staphylococcus* than salt broth was associated with the liquid part of the medium. The liquid was removed and tested in comparison with the whole medium and the broth used in preparing it (section V). Ten per cent. NaCl was added to each. The liquid from the meat and the meat medium itself behaved similarly and both were less inhibitory than broth as judged by the rate of growth of small inocula. Thus, during autoclaving of the medium, there was extracted from the cooked meat something which, like peptone, counteracted the inhibitory effect of NaCl.

*Thioglycollate.* The addition of 0.1 per cent. sodium thioglycollate to plain broth did not alter its ability to grow staphylococci but NaCl was much more inhibitory in the presence of thioglycollate (section V). The effect of NaCl would thus appear to be enhanced by an increased reduction potential.

*Anaerobiosis.* Direct observations were made by incubating cultures of *Staphylococcus* in broth and on agar, in Robertson's meat medium and thioglycollate medium, with and without 10 per cent. NaCl, in an anaerobic jar and in air. Anaerobic conditions alone had a slight retarding influence on the growth of *Staphylococcus* in the ordinary media. In salt media this influence was a little more pronounced but whether there was a summation of the effects of anaerobiosis and NaCl or whether anaerobiosis might have acted indirectly requires further investigation.

## DISCUSSION

By using a method to reveal quantitative differences and comparing several media we have shown that *Staphylococcus* is much more resistant to the growth-inhibitory action of NaCl than most other bacteria derived from man and that the composition of the medium has a considerable influence on this action. There is possibly some indication (section III) that the nature of the action of NaCl may be similar for different species but may operate at different concentrations; *i.e.* the same gradation of effect on growth was produced for different organisms at different ranges of salt concentration. This is however no more than a suggestion and further investigation is required to elucidate the nature of the action of NaCl and of substances which have been shown to diminish its effect.

## SUMMARY AND CONCLUSIONS

1. Robertson's meat medium with 10 per cent. of added NaCl is the selective medium of choice for isolating staphylococci. It will suppress the growth of most other bacteria and will detect a small number of staphylococci, calculated as about 10, in a large mixed inoculum.
2. Meat medium is less inhibitory to staphylococci than broth or agar with 10 per cent. added NaCl, and much less inhibitory than digest broth or Brewer's thioglycollate medium containing 10 per cent. added NaCl.
3. Substances which diminish the inhibitory effect of 10 per cent. NaCl on the growth of staphylococci are present in peptone, serum, blood extract and the liquid part of Robertson's meat medium.
4. In practice 10 per cent. of NaCl added to Robertson's meat medium proved highly satisfactory for isolating staphylococci from mixed inocula such as faeces, mouth washes, milk and ice-cream. Enrichment in this medium by incubation overnight followed by plating yielded pure or almost pure cultures of staphylococci.

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## ACTINOMYCOSIS IN DOGS

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(PLATES XCV-XCIX)

SEVERAL cases of actinomycosis in the dog and cat were seen in this department during the last two years. The distribution and type of the lesions varied considerably in different cases, ranging from liver abscesses in a six-weeks-old puppy to widespread lesions with peritonitis in older animals, usually accompanied by abdominal fluid containing numerous sulphur granules. Microscopic examination of the lesions always showed Gram-positive branching filaments.

### LITERATURE

The causal organism of actinomycosis in man and cattle has received much attention, but records of canine actinomycosis are limited in their scope. Rabe (1888, quoted by Lesbouyries, 1942) found in canine lesions a filamentous organism resembling that of bovine actinomycosis. He named it *Cladothrix canis*, and Chalmers and Christopherson (1916-17, p. 255) refer to it under the synonym *Nocardia canis*. Klimmer (1934), in his study of the aetiology of actinomycosis in animals, dealt only with the ox and pig. Baudet (1934) described three cases and suggested that the pathogenic organism should be called *Cohnistrepthothrix canis*. In a case of meningitis in the dog, Balozet and Pernot (1936) demonstrated an organism which they considered identical with what they styled the *Actinomyces asteroides* of Eppinger (1891). Breed, Murray and Hitchens (1948) list *Actinomyces canis* among the ill-defined species of *Nocardia*. Eroms (1939) described two cases in dogs which showed a chronic purulent productive inflammation of the peritoneum and pleura. In both, histopathological examination revealed a picture typical of actinomycosis. Lesbouyries (1942) suggested that the causal agent of canine actinomycosis was a facultative anaerobe, a variant of *Actinomyces israelii*. Martin (1942) described three cases of canine actinomycosis in which the lesions were found in the lungs, liver and abdominal cavity respectively. The histo-pathological picture of the lesions was of fibrous tissue surrounding areas of massive infiltration with polymorphonuclear leucocytes, lymphocytes, histiocytes and other mononuclear cells. In some of the infiltrated areas rosettes were observed with peripheral radiating clubs and granular centres. The disease was normally chronic in nature, but a terminal, rapidly fatal, acute exacerbation was also recorded. In their textbook, Kelser and Schoening (1943, p. 394) wrote: "Of the domestic animals, the bovine species is most susceptible to actinomycosis. The disease is also of relatively frequent occurrence in hogs. Rarely are horses, sheep, and other animals affected."

These effects were reproduced even after repeated subculture. Appearances in other media may be described briefly as follows.

*Agar slope.* Slightly raised white colonies, described above as type 3, grew along the line of inoculation. *Gelatin.* Stab cultures maintained at room temperature showed neither liquefaction nor growth. After 5 days at 37° slight growth without liquefaction was seen. *Shake culture.* In glucose-agar shakes good growth was seen at the surface and slight growth below it; growth was absent from the depths of the medium. *Loeffler's serum* and *Dorset's egg medium.* Good confluent growth without digestion of medium; growth only slightly adherent to medium. *MacConkey's medium.* No growth after 5 days at 37°. *Potato medium.* Growth evident after 18 hours at 37°. Colonies white and evenly raised at first, but later showing brown pigmentation and wrinkling of the surface. A chalk-white bloom developed on the upper parts and sides of the potato during incubation.

*Metabolism.* In many subcultures this organism grew well in both liquid and solid media with the few exceptions already noted. For good growth, the cultures required normal atmospheric conditions; anaerobiosis or replacement of O<sub>2</sub> with CO<sub>2</sub> inhibited growth. The optimum temperature was 37° C., and there was growth only after 72 hours at room temperature.

*Biochemical reactions.* After 30 days at 37° no change was observed in glucose, lactose, mannitol, inositol, maltose, salicin, dulcitol, sucrose, raffinose, arabinose, sorbitol, dextrin, rhamnose, inulin, xylose, trehalose or glycerol. Litmus milk was unchanged after 8 days at 37° and indole was not formed in peptone solution.

*Resistance.* If broth cultures which had already grown were left at room temperature, surface growth which had dried out on the sides of the tubes from evaporation of the culture fluid was found to be viable after 6 weeks. Cultures kept for 8 weeks at 8° C. showed no loss of viability, and a temperature of 75° had to be maintained for 10 minutes to ensure sterilisation (table I).

TABLE I  
*Heat resistance of organism isolated from case 1*

Exposure		After exposure. Results of incubation at 37° C. for (hours)		
Time (min.)	Temperature (° C.)	24	48	72
5	55	+++	...	...
10	"	+++	...	...
15	"	+++	...	...
5	65	+++	...	...
10	"	+++	...	...
15	"	—	+	++
5	75	—	—	+
10	"	—	—	—
15	"	—	—	—

+, ++, +++ denote increasing amounts of growth.  
— denotes absence of growth.

## ACTINOMYCOSIS IN DOGS



FIG. 1.—Surface type-1 colonies of the organism from case 1 on blood agar after 18 hrs. at 37° C.  $\times 10$ .

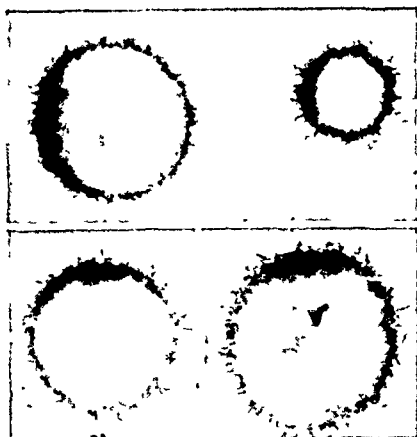


FIG. 2.—Surface type-1 colonies of the organism from case 1 on nutrient agar after 5 days at 37° C.  $\times 13$ .

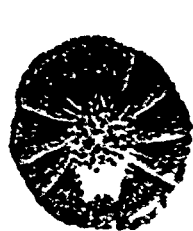
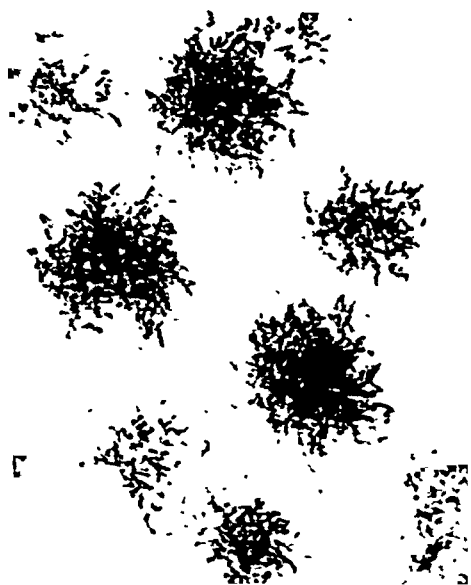


FIG. 3.—Surface type-1 colonies of the organism from case 1 on blood agar after 5 days at 37° C., showing central crater and rosette.  $\times 10$ .

FIG. 4.—Surface type-1 colony of the organism from case 1 on blood agar after 5 days at 37° C., showing star formation  $\times 14$ .







*Pathogenicity*

Mice proved to be entirely resistant to this organism whether they were inoculated intravenously or intraperitoneally. Guinea-pigs and rabbits survived intraperitoneal inoculation and, on post-mortem examination after being killed, showed only minor lesions in the liver. Rabbits proved to be highly sensitive to intravenous inoculation of the organism, as 1 c.c. of a 5-day glucose-broth culture regularly killed them in four days, and post-mortem examination consistently showed numerous disseminated lesions of minute size affecting practically all organs and tissues, with marked dehydration of the subcutaneous tissues.

The lesions, which were small, elongated and greyish-white, appeared to be most numerous in the myocardium, kidneys, fundus of the gall bladder and intestinal wall. Numerous similar lesions were noted in the musculature, liver, spleen and lymph glands. Crushing of the foci released branching filaments which were acid-fast and Gram-positive (fig. 8). Cultivation of the organisms from the tissue lesions gave the same results as those described for the original isolation.

*Histopathology.* Histological examination of the lesions showed a structureless disintegrated centre surrounded by a zone of infiltration by leucocytes and mononuclear cells with many proliferating fibrocytes (fig. 11).

*Effect of penicillin.* Two rabbits received an intravenous injection of 1 c.c. of a 5-day broth culture at 37° C. plus 8000 units of penicillin. These animals showed no ill effects from the inoculation and were killed after 30 days. Post-mortem examination showed no lesions except a few foci in the cortex of the kidneys and in the liver. Smear examination of these foci failed to reveal the presence of organisms and histological examination showed that the cortical lesions in the kidneys were of a chronic nature (fig. 12).

**Case 2**

*Clinical history.* A 15-months-old Labrador dog showed signs of abdominal pain, dullness, anorexia and progressive emaciation. As the condition did not respond to treatment, and palpation showed the presence of what appeared to be a tumorous mass, the animal was destroyed.

*Post-mortem findings.* The abdominal cavity contained about half-a-pint of turbid reddish-brown fluid with numerous yellow-white floccules. The omentum and mesentery were grossly thickened and hard to the touch, and on being cut into showed much fibrous tissue surrounding greyish-yellow foci. The mesenteric lymph glands were enlarged and hyperæmic. Diffuse peritonitis was present and the liver and spleen both showed congestion. Smears from the granulomatous tissue showed the presence of non-acid-fast, Gram-positive branching filaments.

*Histological examination* showed numerous areas of leucocytic infiltration, with mononuclear cells and a few giant cells. The infiltrated areas were separated by bands of fibrous tissue (fig. 13).

### *Isolation of the organism*

Inocula from foci in the granulomatous tissue were smeared on solid and inoculated into fluid media, all being incubated at 37° C. under aerobic and anaerobic conditions. Some glucose-broth cultures incubated for 24 hours aerobically showed one or two floccules at the bottom of the tube, others had no visible growth. After 5 days at 37° the cultures showed either a scanty growth at the bottom of the tube or colonies suspended in the fluid; the broth remained clear. Growth in nutrient broth was even poorer than that in glucose broth, but enrichment with serum improved it. Nutrient agar showed very scanty growth.

*Blood agar.* After 24 hours at 37° C., numerous colonies developed, their size ranging from 1 to 2 mm. in diameter. After 6-8 days at 37° the mean diameter of colonies had reached 10 mm. and in some it was as much as 15 mm.

Slight pitting of the medium was noted, and on the raised surface of the colonies, dull and dark at first, there appeared a white apical spot from which filaments developed. These formed a white bloom which spread over the whole colony. Only a narrow translucent margin remained free for several days (figs. 14 and 15). The central elevation usually seen in young colonies (fig. 16) was later replaced by a central crater (fig. 17), though in some cases the crater developed before any protuberance was observed. The white bloom was easily removed with a loop, exposing a slightly convex colony with a shiny gelatinous appearance and of leather-like consistency. The colonies adhered firmly to the medium and developed a dough-like consistency if kept in the refrigerator for some time. As they aged, cultures emitted a strong mouldy smell.

*Hæmolysis.* Colonies on horse-blood agar plates incubated for 24-48 hours at 37° C. showed a broad zone of hæmolysis, followed by clearing of the whole plate.

*Other media.* *Agar slope.* After 72 hours at 37° only very sparse growth. *Gelatin.* Neither growth nor liquefaction after 21 days. *Shake culture.* A few single surface colonies in glucose agar after 72 hours at 37°. *Loeffler's serum.* Slight growth after 72 hours at 37°. Colonies reached a mean diameter of 10 mm. in 5-6 days. Commencing liquefaction of the serum was noted after 72 hours at 37° and was almost complete in 21 days. *Dorset's egg medium.* Good confluent growth with softening of the medium. *MacConkey's medium.* No growth. *Potato medium.* No growth after 21 days at 37°.

*Morphology.* Stained smears from the various cultures and from the lesions showed non-acid-fast, Gram-positive branching filaments. A tendency to terminal club formation was noted. No breakdown

ACTINOMYCOSIS IN DOGS

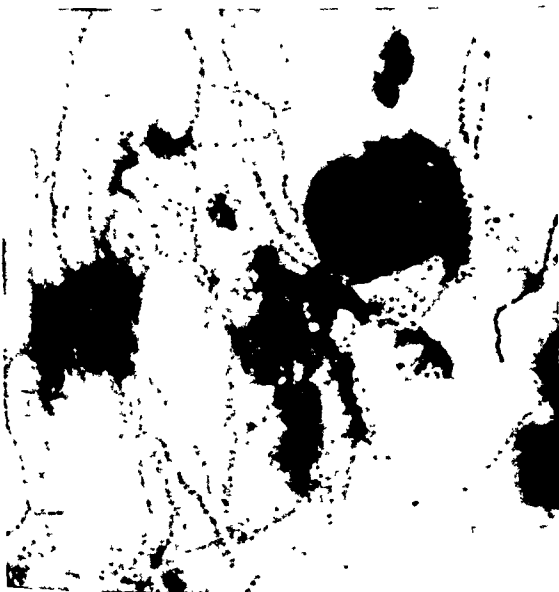


FIG. 7.—Case 1. Smear from sinus discharge, showing branching and beaded filaments. Gram's stain.  $\times 800$ .



FIG. 9.—Organism from case 1. Glucose-broth culture after 6 days at 37° C. Gram's stain.  $\times 730$ .



FIG. 8.—Organism from case 1. Kidney lesion of inoculated rabbit, showing branching and beaded filaments. Gram's stain.  $\times 800$ .



FIG. 10.—Organism from case 1. Breakdown forms in glucose-broth culture after 14 days at 37° C. Gram's stain.  $\times 730$ .



ACTINOMYCOSIS IN DOGS



FIG. 11.—Lesion in kidney of rabbit inoculated with 1 c.c. of virulent culture.  
Hæmalum and eosin  $\times 200$ .



FIG. 12 Lesion in the cortex of the kidney of a rabbit inoculated simultaneously  
with culture and penicillin. Hæmalum and eosin.  $\times 200$ .



ACTINOMYCOSIS IN DOGS

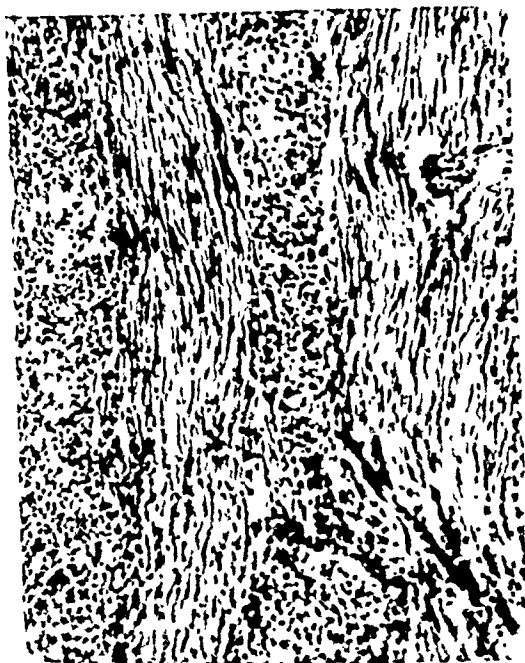


FIG. 13.—Case 2. Omental lesion from dog. Haemalum and eosin.  $\times 200$ .



FIG. 14.—Organism from case 2. Surface colony on blood agar after 24 hrs. at  $37^{\circ}\text{C}$ ., showing translucent halo.  $\times 4$ .



FIG. 15.—Organism from case 2. Surface colonies on blood agar after 48 hrs. at  $37^{\circ}\text{C}$ . Translucent halo nearly covered by spreading filaments.  $\times 4$ .



FIG. 16.—Organism from case 2. Surface colonies on blood agar after 72 hrs. at  $37^{\circ}\text{C}$ ., showing (above) a central protuberance.  $\times 7$ .





## ACTINOMYCOSIS IN DOGS



FIG. 17.—Organism from case 2. Surface colony on blood agar after 6 days at 37° C., showing central cleft.  $\times 6$ .

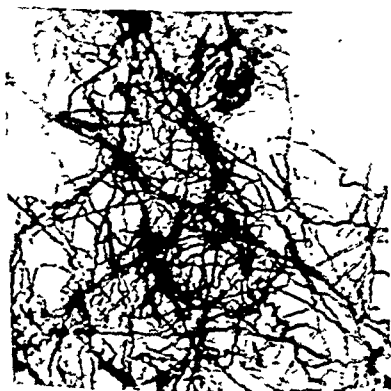


FIG. 18.—Case 2. Smear from omental lesion showing branching filaments. Gram's stain.  $\times 730$ .



FIG. 19.—Organism from case 2 in glucose-broth culture after 14 days at 37° C., showing branching filaments with rudimentary club formation.  $\times 900$ .



forms were found (figs. 18 and 19). Examination for motility was negative.

*Metabolism.* Growth was poor in liquid media and on nutrient agar but satisfactory on blood agar and Dorset's egg medium. Anaerobiosis or replacement with  $\text{CO}_2$  inhibited the growth. Optimum temperature  $37^\circ \text{C}$ . No growth at room temperature after 21 days.

*Biochemical reactions.* After 30 days at  $37^\circ$ , no change in glucose, lactose, mannitol, inositol, maltose, dulcitol, salicin, sucrose, raffinose, arabinose, sorbitol, dextrin, rhamnose, inulin, xylose, trehalose or glycerol. Litmus milk unchanged. No growth could be demonstrated after 6 and 14 days at  $37^\circ$ . Indole was not formed in peptone solution.

*Resistance.* Cultures on blood agar and Dorset's egg medium showed no marked reduction in viability after 30 days. Although resistant to refrigerator temperature, the organism was killed by exposure to a temperature of  $60^\circ \text{C}$ . in 10 minutes (table II).

TABLE II

*Heat resistance of organism isolated from case 2*

Exposure		After exposure. Results of incubation at $37^\circ \text{C}$ . for (hours)		
Time (min.)	Temperature ( $^\circ \text{C}$ .)	24	48	72
5	55	+	++	+++
10	"	—	+	++
15	"	—	+	++
5	60	—	+	+
10	"	—	—	—
15	"	—	—	—
5	65	—	—	—
10	"	—	—	—
15	"	—	—	—

+, ++, +++ denote increasing amounts of growth.

— denotes absence of growth.

*Penicillin sensitivity.* By the ditch-plate method the organism was found to be as sensitive to penicillin *in vitro* as a hæmolytic staphylococcus.

*Pathogenicity.* Five-day cultures in doses up to 5 c.c., if injected intraperitoneally or intravenously, failed to produce any reaction in rabbits and guinea-pigs. Lesions could not be demonstrated in any of the experimental animals, which were killed and examined after 30 days' observation.

## DISCUSSION

Eppinger in 1891 described a filamentous organism in a cerebral abscess of man. He called it *Cladothrix asteroides* because of its branching, but MacCallum (1902), who described another case in a

child, proposed the name *Actinomyces asteroides* in place of *Cladothrix asteroides*. The organism described by these authors grew well aerobically but did not develop under anaerobic conditions. Colonies on solid media showed a central crater-like depression or were elevated into irregular folds. The development of filaments gave the colonies a dull and roughened appearance. The organism did not liquefy gelatin; in broth it formed a granular pellicle without turbidity. On potato, chromogenic growth was recorded with the later development of a white chalky bloom. Smears showed Gram-positive filaments and the organism proved highly pathogenic to rabbits, intravenous inoculation of 1 c.c. of culture causing death in 4 days. At post-mortem examination abscesses were noted in most organs and tissues. Neither Eppinger nor MacCallum mentioned whether the organism was acid-fast. Nothing is said of fermentation of carbohydrates or of hæmolytic properties.

Other cases of human infection with *Actinomyces asteroides* have been recorded by other workers, but to the best of our knowledge this *Actinomyces* has only once before been isolated from a dog—by Balozet and Pernot from a case of canine meningitis. The cultural characteristics of their organism correspond to those of Eppinger's and MacCallum's. They emphasised its non-fermentation of carbohydrates and the finding that 1 c.c. of a broth culture by the intravenous route killed rabbits in four days. The details correspond very closely with those for the organism we isolated from case 1.

The strain of *Actinomyces* isolated from case 2 proved non-pathogenic to laboratory animals, but having eliminated the possibility of other causal agents we felt bound to nominate it as the cause of the granulomata found in the Labrador. Martin and others give histopathological pictures similar to ours for lesions of actinomycosis in the dog. This particular strain of *Actinomyces* cannot be identified as belonging to any of the recognised species.

Some authors suggest different classifications for the causal agent of actinomycosis in dogs. Baudet favours the name *Cohnistreptothrix canis* because the organisms he encountered resembled the morphology of *Cohnistreptothrix israelii*. Eroms, after a histopathological study of two cases, cited the opinion of another department, which carried out the bacteriological examination of the dogs, that the organism was a Wolff-Israel-like *Actinomyces*. Eroms found it difficult to decide if his causal organism was a Wolff-Israel strain or some canine variant.

In the light of our observations in this paper it is difficult to accept the organisms named in earlier studies on dogs as a guide to what we should call the strains now described. Neither of our strains has the features mentioned by Baudet, Lesbouyries or Eroms. Diphtheroid, rod and coccal forms are found in smears from cultures, but if Erikson (1940) is correct these forms represent "breakdown products," which on inoculation into an animal once more give rise to long branching

filaments. Wilson and Miles (1946) give good reasons for not applying the terms *Streptothrix* and *Nocardia* to organisms of the genus *Actinomyces*.

The organisms isolated from both our cases are strict aerobes, but they differ in their morphological, cultural and pathogenic characteristics. The organism from case 1 shows breakdown products, is acid-fast, does not liquefy Loeffler's serum, grows well on potato, is slightly hæmolytic and highly pathogenic to rabbits. It closely resembles in all its features the *Actinomyces asteroides* of MacCallum (= *Cladothrix asteroides* of Eppinger), which cannot be described as *Cohnistreptothrix hominis* or as a variant, either human or canine, of *Actinomyces israelii*.

The organism from case 2 does not show breakdown products but preserves its long branching filaments on artificial media. It is non-acid-fast, liquefies Loeffler's serum, does not grow on potato, has marked hæmolytic properties and is non-pathogenic to laboratory animals. Its characteristics differ not only from those of the organism of case 1 but also from the features described by both Baudet and Lesbouyries for their organisms.

The sensitivity of the organisms to penicillin is worthy of note. In case 1, 250,000 units of penicillin brought about recovery from the infection and the simultaneous inoculation of as small a dose as 8000 units along with 1 c.c. of virulent culture into rabbits so reduced the effects of the organism that the rabbits survived and were in good health and free from lesions when killed 30 days later. Similar results have been reported in human infections, for the application of 5,800,000 and 5,200,000 units of penicillin in two cases reduced the size of the lesions (Hamilton and Kirkpatrick, 1945). The organism of case 2 was shown by the ditch-plate method to have the same degree of sensitivity to penicillin as a control strain of virulent hæmolytic staphylococcus, but it could not be tested *in vivo* since it was not pathogenic for any of the laboratory animals tested.

#### SUMMARY

A Gram-positive aerobic organism was isolated from the sero-purulent discharge from a throat sinus in a dog (case 1). The organism was found as a dense mycelium of acid-fast filaments which showed true branching; it proved highly pathogenic to rabbits.

The simultaneous injection of penicillin and culture into rabbits neutralised the pathogenic effects of the organism.

The organism closely resembles the *Actinomyces asteroides* of MacCallum (= *Cladothrix asteroides* of Eppinger).

A second Gram-positive aerobic organism was isolated from a large granulomatous growth in the abdomen of a dog suffering from diffuse peritonitis (case 2). This organism formed long filaments which showed true branching; it was non-acid-fast, liquefied Loeffler's

serum and was non-pathogenic to laboratory animals. It was sensitive to penicillin *in vitro*.

We wish to acknowledge the kind co-operation of Messrs J. D. Loughran of Stockton-on-Tees and E. G. Wood of Edinburgh, who supplied both subjects and case histories. We also gladly thank Dr J. S. Garside and Professor A. Robertson for facilities and the opportunity to carry out the work. The photographs are by Mr R. Hood, senior technician, to whom our particular thanks are due.

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616.36—099 : 547.556.31 (*p*-dimethylaminoazobenzene)

## THE GLYCOGEN CONTENT OF RATS' LIVERS AFTER POISONING WITH LARGE DOSES OF *p*-DIMETHYLAMINOAZOBENZENE

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In a previous paper, Orr and Price (1948) described the histological appearances which result from the action of *p*-dimethylaminoazobenzene (DMAB or butter yellow) upon the livers of rats. The dye was administered by the intraperitoneal injection of a solution in lard (37° C. filtrate). Among the effects noted histologically was the absence of glycogen from the livers of rats receiving a single large dose.

The most striking qualitative metabolic difference between normal liver tissue and the tumours derived from it is that in the former the glycolysis is a breakdown of glycogen and in the latter a breakdown of glucose (Orr and Stickland, 1941; Dickens and Weil-Malherbe, 1943). The disappearance of glycogen from the liver as a result of the administration of large doses of DMAB might throw some light on the metabolic changes and therefore must be studied more closely.

Liver poisons in general (*e.g.* phosphorus, chloroform and carbon tetrachloride) are known to lead to depletion of the liver glycogen and this is regarded as evidence of loss of power to synthesise glycogen from glucose. In poisoning with DMAB, however, it is not valid to attribute the absence of glycogen to a defect in the synthetic power of the liver, since it was observed that the treated rats had very poor appetites, and clearly glycogen cannot be expected to be formed in the liver unless the rate of ingestion of carbohydrate, in the period just before the observation is made, exceeds the rate of utilisation. The purpose of this paper is, therefore, to establish beyond reasonable doubt that the liver of rats poisoned with DMAB is unable to effect the synthesis of glycogen even when ample glucose is presented to it.

Our first experimental approach was to feed large amounts of carbohydrate, in various forms, to rats by stomach tube, and to follow the blood sugar level for some hours, after which the animal was killed and the glycogen content of the liver determined. If the liver was failing to remove glucose from the blood, the glucose tolerance of the animals would be decreased, and high blood-sugar values



might be expected. In a number of DMAB-poisoned animals such high blood-sugar values were observed, associated with a low liver glycogen, but not in all. In some cases the dehydration of the blood associated with osmotic distension of the stomach made the blood sugar figures suspect.

It therefore appeared desirable to confirm, by independent methods, that adequate ingestion and absorption of carbohydrate were in fact taking place. The experiments are described in chronological order.

### *Material and methods*

Mixed stock rats were used of an average weight of 211 g. (range 90-300 g.). During experiments the rats were marked and kept in pairs in wire cages. The diet was of experimental importance, and is given separately for each experiment.

Intraperitoneal injections of 2 per cent. DMAB in lard (37° C. filtrate) were used throughout. Dosage is expressed in mg./kg. body weight. Stomach feeding was carried out by using a mastoid retractor as gag and soft rubber catheters (size 3-5) lubricated with glycerol. The solution was then gently injected with a syringe at a rate of 1 ml. in 7-10 seconds. In the early stages there was an occasional fatality due to the tube entering the trachea. After a little practice these accidents ceased and it was found possible to feed rats, with no apparent ill effects, several times a day.

At the conclusion of each experiment the rats were killed by a sharp blow on the back of the head. The liver was extracted immediately and the glycogen estimated by the method of Hynd and Rotter (1930). Blood sugar was estimated according to the method of Hagedorn and Jensen (1923), and other sugar determinations by that of Hanes (1929).

### *Experimental observations*

**Expt. 1.** Rats were injected intraperitoneally with 200 mg./kg. DMAB. The control animals were in two groups. One group received injections of lard filtrate and the other no treatment. The animals were fed on a cake made of equal quantities of oatmeal and cane sugar. The ingredients were mixed together with a little water, compressed into cubes and dried at room temperature. A 4 per cent. aqueous cane sugar solution was given to drink. Records of the weight of food eaten and fluid drunk were kept. Half the rats in each group were killed exactly 24 hours after the injection and the remainder at 48 hours. The glycogen content of the livers is shown in table I. It will be seen that the control animals have considerably more glycogen stored in their livers than the poisoned animals, especially after 48 hours. This was not entirely unexpected, as the histological changes are more marked after the greater period of time. The discrepancy between the weight of food consumed by the groups caused no surprise in view of the poisoning of the experimental animals, but raised the objection that the low liver-glycogen values might be due to their poor appetites.

**Expt. 2.** The rats were starved for 18 hours in order to use up

their stores of glycogen. The fasting blood sugar was estimated. One group of rats received 200 mg./kg. DMAB, one group lard filtrate and one group no treatment. Forty-one hours after the injections,

TABLE I

*Liver glycogen values of control and DMAB-treated rats given carbohydrate ad lib.*

	Group	Time in hours after injection	Food (carbohydrates) consumed (g.)	Fluid drunk (ml.)	Liver glycogen (per cent.)	
						Average
Experimental	DMAB 200 mg./kg.	24	$\left. \begin{array}{c} <1 \\ 5 \\ 3.4 \end{array} \right\}$	$\left. \begin{array}{c} 10 \\ 10 \\ 10 \end{array} \right\}$	2.7	2.5
	"				3.1	
	"				1.8	
	"				1.2	
	"				3.9	
Control	"				2.3	
	1 ml. lard	24	$\left. \begin{array}{c} 40 \\ 35 \end{array} \right\}$	$\left. \begin{array}{c} 25 \\ 25 \end{array} \right\}$	6.4	5.9
	"				5.8	
	No treatment				5.0	
	"				6.5	
	"					
Experimental	DMAB 200 mg./kg.	48	$\left. \begin{array}{c} 10 \\ 3.4 \\ 10 \end{array} \right\}$	$\left. \begin{array}{c} 0 \\ 0 \\ 10 \end{array} \right\}$	4.6	0.8
	"				0.09	
	"				0.05	
	"				0.13	
	"				0.15	
Control	"				0.05	
	1 ml. lard	48	$\left. \begin{array}{c} 40 \\ 60 \end{array} \right\}$	$\left. \begin{array}{c} 10 \\ 24 \end{array} \right\}$	5.6	6.9
	"				7.1	
	No treatment				8.6	
	"				6.3	
	"					

all rats received, by stomach tube, 5 ml. of 20 per cent. w/v glucose. It was estimated that 4 hours would be needed for the absorption of this amount (Cori, 1925). The blood sugar was estimated  $1\frac{1}{4}$  hours later. A second feed of 10 ml. of 20 per cent. glucose was given  $44\frac{1}{2}$  hours from the start of the experiment and  $3\frac{1}{2}$  hours after the first stomach feed. One and a quarter hours after this feed the blood sugar was again estimated, and again at  $48\frac{1}{2}$  hours—4 hours after the second feed. The animals were then killed and samples of liver taken for estimation of the glycogen content (table II). The DMAB-poisoned animals showed very little glycogen in the liver and the blood sugar rose to higher levels in these animals than in the controls.

An attempt was made to obtain the phenomenon with doses of less than 200 mg./kg. of DMAB, because with a higher survival rate there would be less reason to suspect that the liver findings were merely the expression of a general depression of vital processes



in sick or moribund animals. It was also desirable to show that these animals were effectively starved after poisoning, in that both experimental and control animals had low liver-glycogen values.

Expt. 3. Rats received doses of 200, 100 and 50 mg./kg. DMAB after a period of starvation followed by fasting blood-sugar estimations.

TABLE III

*Liver glycogen in starved rats poisoned with varying doses of DMAB*

Time-table (hours)	-24 to 0	0		0	24	
Nature of investigation or treatment	Starve	Fasting blood sugar (mg./100 ml.)	Average	Injection	Liver glycogen	Average
Rat no.						
4	...	59	73	200 mg./kg. DMAB	0.11	0.08
5	...	85		"	0.06	
6	...	74		"	0.08	
9	...	...	110	100 mg./kg. DMAB	1.45	0.9
10	...	110		"	0.35	
13	...	106	100	50 mg./kg. DMAB	0.09	0.45
14	...	94		"	0.81	
19	...	97	82	Control	0.69	0.53
20	...	67		"	0.46	

TABLE IV

*Liver glycogen in rats poisoned with varying doses of DMAB, starved, and then given a known amount of glucose*

Time-table (hours)	-24	0		0	44	48		48	
Nature of investigation or treatment	Starve	Fasting blood sugar (mg./100 ml.)	Average	Injection DMAB	Starve	1.5 g. glucose by stomach tube	Blood sugar (mg./100 ml.)	Average	Liver glycogen (per cent.)
Rat no.									
1	...	83	93	Control	...	...	97	111	2.35
2	...	110		"	...	...	115		6.05
3	...	87		"	...	...	122		2.4
7	...	87	98	100 mg./kg.	...	...	115	102	3.2
8	...	115		"	...	...	106		0.94
11	...	78		"	...	...	90		2.35
12	...	106		"	...	...	96		3.1
15	...	103	86	50 mg./kg.	...	...	104	100	3.5
16	...	92		"	...	...	92		2.35
17	...	80		"	...	...	85		1.24
18	...	69		"	...	...	117		1.78

The usual controls were used. Each group of animals was then split into two batches. The first batch was starved for 24 hours, then

Expt. 6. Rats poisoned with 200 mg./kg. DMAB were starved for 24 or 48 hours. They were treated as in expt. 5 and the results, after one hour's absorption, are shown in table VIII. Adequate absorption of glucose appears to have taken place.

TABLE VIII

*Absorption, after one hour, of glucose from intestine of DMAB-treated rats (200 mg./kg.)*

Rat no.	Duration of preliminary starvation	Glucose administered (mg.)	Glucose remaining (mg.)	Glucose absorbed (mg.)	Liver glycogen (g. per cent.)
1	24	262	156	106	0.13
2	24	249	51	198	0.23
3	24	331	278	53	0.21
4	24	303	212	91	0.27
5	48	280	73	207	0.32

In view of the large gastric residue found in DMAB-treated animals, it was thought desirable to observe the rate of emptying of the stomach directly by X-ray examination. Barium was administered by stomach tube, and X-ray pictures were taken after 1, 3, 6 and 24 hours. It was found that the stomach emptied in about 6 hours in a healthy rat, but required somewhat longer in a DMAB-treated animal. In both cases, there was barium throughout the small intestine after 6 hours.

Expt. 7. This experiment (table IX) was designed to show that the phenomenon persisted when the food intake was raised to the highest practicable level. The animals, after 200 mg./kg. DMAB, received

TABLE IX

*Liver glycogen in rats with very large carbohydrate intake*

Rat no.	Treatment	Liver glycogen per cent.	Average
1	200 mg./kg. DMAB	1.02	1.48
2		1.02	
3		2.06	
4		1.37	
6		1.94	
7	Control	6.70	5.36
8		3.16	
9		2.59	
10		6.13	
11		6.63	
12		6.98	

food and drink *ad lib.* for 48 hours before they were killed for estimation of the glycogen content of the liver. In addition each animal was stomach-fed twice a day with 6 ml. of a mixture of starch 300 g.,

cane sugar 60 g. and water to 600 ml. The liver glycogen of the five experimental animals ranged from 1.02 to 2.06 (average 1.48) per cent. as compared with a range of 2.59 to 6.98 (average 5.36) per cent. in six control animals.

Experiments were then undertaken to see if a similar effect was present after poisoning with carbon tetrachloride. It was not found possible to correlate the survival time with an injected dose expressed in mg./kg. Most erratic results were obtained. The administration of carbon tetrachloride by open anaesthesia gave more satisfactory results. Rats anaesthetised for  $1\frac{1}{2}$  hours survived for 36-48 hours. Rats anaesthetised for less than this time survived. Anaesthesia for 70 minutes was chosen as being 0.8 of the "minimum lethal time", a dose comparable *mutatis mutandis* with 200 mg./kg. DMAB.

Expt. 8. Six rats were anaesthetised for 70 minutes with laboratory re-distilled carbon tetrachloride. They received a diet and stomach feeding exactly as in expt. 7. The liver glycogen was reduced in some cases (range 0.56 to 4.24, average 2.07 per cent.), but the effect did not appear to be more marked than that of DMAB.

### Summary

A series of experiments is described showing that in rats poisoned with single large doses of *p*-dimethylaminoazobenzene (DMAB or butter yellow) the liver failed to lay down glycogen in spite of the ingestion and absorption of adequate amounts of carbohydrate.

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TUBERCULOSIS IN A WILD SPARROW-HAWK  
(*ACCIPITER NISUS NISUS*)

JAMES M. HARRISON

Sevenoaks

(PLATES C AND CI)

On 8th November 1946, the late Dr A. McMillan of New Romney kindly sent me an adult male sparrow-hawk (*Accipiter nisus nisus* Linnaeus), which had been found dead by Major C. F. Krabbé in Dymchurch, Kent. Its right knee-joint was lumpy and the bird was very wasted, weighing only  $3\frac{3}{4}$  oz. instead of the average normal  $5\frac{1}{4}$  oz. The presence of some worn and very faded tail feathers suggested a missed moult. There was a small ulcer about the middle of the keel of the sternum, probably caused by the weakened bird's reclining on this structure.

*Post-mortem appearances*

The bird was quite devoid of subcutaneous fat, and the pectoral muscles were markedly reduced in bulk. The left pectoralis major was infiltrated by growth, and similar greyish-yellow nodular lesions were seen in the left deltoid and left axilla. There was also infiltration of the cervical lymph glands. The right knee showed a large granulomatous mass, extending on to both the medial and lateral aspects of the leg, and in the tibialis anticus just above the tibio-tarsal joint there was a further small lesion.

Within the thoraco-abdominal cavity, multiple lesions were found as follows. *Pro-ventriculus*. The distal third had three lesions, one on the lesser and two on the greater curvature near the pyloric end. *Liver*. The organ was studded throughout with greyish-yellow lesions, ranging from a mere speck to 2 mm. or so in diameter. *Lungs*. The left lung was so largely destroyed by nodular deposits that only a little bright red lung tissue was visible in the hilar region. The pathological process had presumably extended from the lung via the air-sac system to the parietal surface of the sternum and thence to the left axilla, where the large penetrating mass was observed. It had not, however, extended into the ostia of the sternal keel. It was apparent that the muscular, bony and articular structures of the left pectoral girdle were grossly involved. *Pericardium*. This



showed three small nodules. There was no macroscopic evidence of involvement of the spleen, pancreas or uro-genital system, or of the rest of the alimentary tract or mesentery.

In birds, it is generally supposed that widespread tuberculosis, as this appeared to be, has its origin in an alimentary tract infection, presumably from the ingestion of an infected quarry. The three lesions in the pro-ventriculus would therefore seem to represent the primary focus. The absence of intestinal lesions is remarkable, in view of the extensive dissemination throughout other parts of the portal circulation. The liver is always grossly affected. Dissemination by lymphatics can account for lesions in the parietal pleura and pericardium, and peri-articular lesions may represent infection from the blood stream.

### *Joint tuberculosis*

Joint tuberculosis has often enough been noted in poultry, but this is the first example I have seen in a limited experience of tuberculosis in wild birds. Accordingly I had X-ray photographs taken of the bony lesions, with corresponding views of these parts in a strictly comparable adult male sparrow-hawk.

The antero-posterior view of the affected leg (fig. 1) in contrast to that of the normal (fig. 2) shows the clear outline of the dense fibro-granulomatous peri-articular mass and the lateral (varoid) displacement of the knee-joint resulting from the deformity. There is obvious decalcification with marked attenuation and bending of the fibula and the bone has shortened from atrophy and absorption. Necrosis at the tibio-fibular joint, the point at which the peri-articular infection was released, has led to upward dislocation of the fibula. There is also loss of the normal cancellous structure of the lower end of the femur. A lateral view of the infected and normal knee-joints showed that both patellæ were of the same dimensions—a point of interest in that the patella of a tuberculous joint in the human subject is not infrequently larger than normal.

In the X-ray of the affected left shoulder (fig. 3), the following appearances are noted in contrast to the normal appearance of the same area (fig. 4). The outline of a fibro-granulomatous peri-articular mass is seen, similar to that around the knee-joint. The cancellous tissue of the head of the humerus has suffered some destruction and shows a tendency to become cystic. The rather "fuzzy" shadows of the upper part of the humeral head suggest necrosis, and, although it is not so clearly seen as in the knee-joint, there is a point on the supero-medial aspect of the head where the infection has discharged into the soft tissues. Another such point, where the cortex of the humerus has been breached by the inflammatory process, is to be seen on the supero-lateral aspect, where a well-defined cyst has formed and burst. These two points represent the ostia through which infection of the peri-articular and soft tissue has taken place.









These characters are very similar to the radiological appearances of the human tuberculous joint, though the porous and cancellous nature of avian bones alters the picture, and gives an appearance of fibro-cystic disease which is rarely evident in the human subject.

The tuberculous reaction in the bone itself is therefore not very different in bird and man, but there is nothing in human tuberculosis akin to the firm, fibrous and nodular formations around the joints, which are perhaps the most typical feature of the disease in birds.

### Histology

Sections from the pro-ventriculus, liver, lungs, and peri-articular mass in relation to the right knee-joint were stained with hæmatoxylin and eosin and by Ziehl-Neelsen's method for acid-fast bacilli. Photomicrographs of the sections of the pro-ventriculus (figs. 5 and 6) and of the liver (figs. 7 and 8) show the nature of the lesions and the presence of an organism resembling *Mycobacterium tuberculosis*.

### Discussion

Harrison (1946) quoted nine investigated cases of avian tubercle in four species of wild birds: six in the pheasant (*Phasianus colchicus*) and one each in a short-eared owl (*Asio flammeus*), a black-headed gull (*Larus ridibundus*) and a starling (*Sturnus vulgaris*). In these, the lesions were most often found in the liver, spleen, œsophagus, pro-ventriculus, gizzard and intestine—a marked predominance of infection in the abdominal viscera. This clearly points to the alimentary tract as the usual portal of entry of the infection in birds. Harrison (p. 204) cites the following species as having been found with the disease: gull, starling, wood-pigeon, sparrow, lapwing and chaffinch, and quotes Mitchell and Duthie (1929) as recording 15 per cent. of 40 crows as tuberculous. Rooke (1946) reported the disease in the barn-owl (*Tyto alba*). In 1933, Kalter *et al.* noted the infection in a male eastern sparrow-hawk, and Hare (1931-32) recorded the disease in a blackbird. Most of the infected species are either scavengers or predators, which would naturally be ready to seize so easy a prey as a weakened tuberculous quarry.

The case described in this paper is of interest in that the disease was widespread and included multiple bony and ligamentous lesions. Visceral tuberculomata in birds are well known, and their histology is typically tuberculous. Broadly, this shows the usual concentric zones around a necrotic eosinophil mass of debris resulting from destruction of the host's tissue cells. Next is a layer of epithelioid cells of varying depth, with plasma-cells, lymphocytes and some fibroblasts, the innermost cells imperfect, structurally damaged and partly lysed, the peripheral cells better defined. Vacuolation of cells is seen in this layer, and in its outer zone many giant-cell systems

are seen. Outside this there is a well-defined zone of strongly basophil lymphocytes, and lastly, at the periphery of the tubercle, a fibrocytic layer of variable depth—evidence of a reaction limiting the spread of infection.

These lesions are clear-cut and discrete in the liver, where they are visible to the naked eye, in the histological sections, as intensely stained, minute scarlet dots in an otherwise normal matrix of liver tissue. In the lung, the histological structure is similar, though the lesions are less sharply defined. In the pro-ventriculus the tubercles are situated in the submucosa. In the dense fibrous tissue surrounding the joints, the essential characteristics of the tubercle are modified by the avascularity of the tissue.

### Summary

An acid-fast infection resembling avian tuberculosis caused the death of a wild adult male sparrow-hawk (*Accipiter nisus nisus*). The distribution of the lesions included the liver, lungs and two major joints with their associated ligamentous and muscular structures. The post-mortem and histological appearances are consistent with a tuberculous infection, and the demonstration of acid-fast organisms with morphological characters indistinguishable from those of *Mycobacterium tuberculosis* confirms the suggested diagnosis. The appearances were in keeping with an initial infection by way of the alimentary tract and later spread by the blood and lymphatics and by direct extension.

My thanks are due to the late Dr Arnold McMillan for the specimen; to Dr Hugh R. C. Hay and Miss Stella Ticehurst for the radiographs; to Professor W. G. Barnard, Mr F. J. F. Barrington and Mr K. S. Macdonald for the histology and photomicrographs; and to my son, Dr Jeffery G. Harrison, for the adult male control sparrow-hawk.

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# TOXIC EFFECTS OF SALICYLATE ON THE FŒTUS AND MOTHER

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In January 1948, an eight-months-pregnant woman in the second stage of labour was admitted to University College Hospital suffering from aspirin poisoning. Nine hours before, she had taken 200 g. of aspirin with suicidal intent. She was at first irrational, noisy and hyperpnoëic, but three hours later her condition was "fair" and she was conscious and willing to co-operate. Eight hours after admission she gave birth, by breech delivery, to a dead male child weighing 5 lb. 15 oz. By next day the mother was quiet and rational and except for some tinnitus and headache which persisted for a day or two she experienced no further trouble.

It was thought possible that the child had died, *in utero*, from salicylate poisoning but autopsy revealed tentorial tears and cerebral hæmorrhage without other noteworthy features. However, a sample of blood from the cord was found by Dr John Humphrey to contain a high concentration of salicylate and this finding maintained our interest in the possibility of premature death *in utero* being caused by salicylates. Furthermore, standard pharmacology textbooks contain references to the danger of using large doses of salicylates during pregnancy. Cushny (1947), for instance, says of "Salicylic preparations" that "large doses should be used with care in cases of pregnancy, as they may lead to miscarriage"; according to Davison (1944) "Large doses (of salicylates) are contra-indicated in pregnancy as abortion may result," while McGuigan (1940) remarks "Why hemorrhages, abortions and miscarriages occur after large doses of salicylates is unknown (Binz)." If these warnings are justified they should be more generally known and more widely heeded, since a considerable proportion of the population, and no doubt many pregnant women, take aspirin. On reading the relevant obstetrical literature and on discussing the matter with obstetrical colleagues I gained the impression that there is very little clinical support for these statements. Chorea of pregnancy, for example, is commonly treated without harm to the fœtus with large doses of salicylates. These uncertainties stimulated the following investigation.

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## EXPERIMENTAL OBSERVATIONS

*Placental transmission of salicylate*

Experiments were performed to investigate the probability, *a priori*, that salicylates can pass through the rabbit placenta. Rats proved unsatisfactory for this kind of work because sufficient blood cannot readily be obtained from the small foetuses.

A rabbit, weight 3800 g., in the 30th day of pregnancy, was injected subcutaneously with 3.8 g. (= 1 g. per kg.) of sodium salicylate in aqueous solution. Two hours later heart blood was collected from the mother. At the same time the uterus was removed under light ether anaesthesia and blood obtained from each foetus by cutting the vessels at the apex of the right pleura and collecting it from the pleural sac with a Pasteur pipette. Samples were allowed to clot and the salicylate content (expressed as mg. of salicylic acid per ml. of serum) estimated by the method of Brodie *et al.* (1944). Their technique was followed closely except that the ethylene dichloride-aqueous mixtures were not centrifuged to effect separation but simply allowed to stand in glass measuring cylinders for a few moments until adequate separation had taken place. In this experiment the foetal sera were pooled, as insufficient serum was obtained from each foetus for separate estimation. The maternal serum of this animal contained 0.58 mg. of salicylic acid per ml. and the pooled foetal sera 0.37 mg. per ml.

In another experiment a 4200-g. rabbit in the 30th day of pregnancy was given 6.3 g. (= 1.5 g. per kg.) of sodium salicylate subcutaneously. Two hours later the maternal serum contained 0.75 mg. of salicylic acid per ml. and in five of the foetuses from which sufficient blood was obtained for separate estimations the corresponding values were 0.45, 0.52, 0.50, 0.52 and 0.62 mg. per ml. respectively. These observations agree with those of Fehling (1876, rabbits), Zweifel (1877, women) and Lannois and Briau (1898, guinea-pigs and rabbits).

It is evident that salicylate can pass quite readily through the placental "barrier" in the rabbit, an observation of some clinical interest, for Ratner *et al.* (1927) have shown that the placental permeability of man resembles that of rodents such as rats, rabbits and guinea-pigs.

*Toxicity of salicylate for the foetus*

Since salicylates readily pass through the placenta, experiments were carried out to determine whether doses of this drug not lethal for the mother regularly cause abortion in rats and rabbits. Repeated injections over a prolonged period were not thought advisable, as this procedure leads to large subcutaneous sloughs which become infected, while anorexia may be severe, with reduced intake of food and vitamins. Vitamin-K deficiency may well be a factor in the production of the hæmorrhages and hypoprothrombinæmia which,

as will be noted later, some workers have stressed as important features of salicylate poisoning. Each animal was given a single injection only and the dose for different animals was varied up to and beyond the lethal dose for mothers. After an injection of sodium salicylate a pregnant rat or rabbit either dies in 4-7 hours—even earlier with very large doses—or it survives and is apparently well 24 hours later. All the animals were in the last week of pregnancy. Two days after the injection the mother was lightly anaesthetised, the uterus rapidly removed and the foetuses examined for signs of life such as active wriggling movements in response to light stimulation.

**Rats.** The lethal dose for rat mothers was between 0.5 and 0.75 g. per kg. subcutaneously (table I).

TABLE I

*Proportion of deaths of rat mothers after subcutaneous injection of various doses of sodium salicylate*

Dose (g. per kg.) of sodium salicylate subcutaneously	No. of rats used	No. of survivors	No. dying
0.75	3	0	3
0.50	5	3	2
0.40	2	2	0
0.30	1	1	0
0.20	2	2	0

It was apparent that whenever the mother survived, so did all the foetuses (table II).

TABLE II

*Proportion of foetal deaths after subcutaneous injection of rat mothers with various doses of sodium salicylate*

Dose (g. per kg.) of sodium salicylate subcutaneously to mother	No. of rat survivors	Proportion of surviving foetuses in each litter
0.50	3	9/9 12/12 11/11
0.40	2	10/10 12/12
0.30	1	10/10
0.20	2	10/10 6/6

**Rabbits.** A similar experiment was done using a few rabbits only. The lethal dose of sodium salicylate for pregnant rabbits is between 0.5 and 1.0 g. per kg. (table III).

Of the three surviving mothers in only one were there any foetal deaths; four out of the 13 foetuses found in this animal were dead. In other words, even when the mothers were given a dose of salicylate which killed one out of 4 of them, most of the foetuses survived (table IV).

between hexose diphosphate and pyruvate. The glycogen depletion is readily demonstrable by means of Best's carmine stain 7 hours after the subcutaneous injection of 1 g. per kg. of sodium salicylate into a non-pregnant adult rat.

There is no doubt that Lutwak-Mann has the right approach to the problem. Death from acute salicylate poisoning is due to interference with intracellular enzyme activity and death may ensue before such interference can be manifested by histological changes. The emphasis on the importance of hæmorrhages sometimes seen *post mortem* in salicylate poisoning (and the same comments apply to the stressing of minor lesions in other acute deaths such as acute septicæmia, hypoglycæmic coma, etc.) is due to a not uncommon and perhaps understandable reluctance on the part of the morbid anatomist to admit that the naked eye and the microscope can reveal only the crude and often unimportant lesions which are secondary to essential intracellular changes.

#### SUMMARY

1. Salicylates are transmitted from the mother to the foetus with ease in rabbits and rats, the blood concentrations being similar in the two cases.

2. Salicylates are no more toxic for the foetus than for the mother. There is no special liability to abortion or foetal death in salicylate poisoning until doses approaching maternal lethal levels are reached.

3. Neither hæmorrhages, hypoprothrombinæmia nor increased capillary permeability are found in rabbits or rats dying after a single injection of sodium salicylate.

4. Liver glycogen is seriously depleted in acute salicylate intoxication.

5. The opinion is expressed that there are no specific histological changes in salicylate poisoning and that the harmful effects may be referred to an interference with intracellular enzyme activity.

I wish to thank Prof. W. C. W. Nixon for the case notes and to express my indebtedness to Prof. G. R. Cameron, F.R.S., for his advice and guidance in this work.

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## BACTERIAL VARIATION AND TOXIN PRODUCTION BY *CORYNEBACTERIUM DIPHTHERIÆ*

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THE difficulty of obtaining consistently high yields of diphtheria toxin is a problem long familiar to those engaged in the large-scale production of this important biological substance. Successive batches of culture medium prepared in exactly the same manner and inoculated with the same seed culture may yield toxins differing considerably in potency; and if the individual flasks of any one batch are tested separately wide variations in their toxin content may be discovered. Until recently, no adequate explanation of these irregular results had been found. In the present communication evidence is produced to show that these variations cannot be attributed to slight differences in the media or cultural conditions but that, on the contrary, they are due to intrinsic properties of the strain of *Corynebacterium diphtheriæ* employed. As the investigations progressed it became evident that the results could be interpreted in terms of bacterial variation. They thus fell into line with views recently expressed by other workers in this field, and the conclusions ultimately reached are in full agreement with this concept. By making use of this new knowledge the difficulties and uncertainties of routine toxin production can now be largely overcome.

### PREVIOUS INVESTIGATIONS

The Park-Williams no. 8 strain of *C. diphtheriæ* (P.W. 8), used exclusively in the present studies, was originally isolated by Park in 1895 from a patient suffering from a mild attack of faucial diphtheria. Since that time the strain has been employed in laboratories all over the world for the large-scale production of diphtheria toxin, and for this purpose it still appears to have no equal. From time to time, however, reports have appeared that the strain had deteriorated in toxigenicity. In 1922 Wilcox reported that the original strain maintained at the Laboratories of the New York State Department of Health had ceased to yield toxin of good quality and that it had been necessary to replace it by a sub-strain which Dr Park had given to the Pasteur Institute, Paris, in 1896. Some years later the toxigenic power of the P.W. 8 strain used at the Connaught Laboratories, Toronto, became unstable. The cause of this was investigated by Cowan (1927), who succeeded in isolating a completely non-toxigenic and avirulent variant from the parent strain by a method of serial colony selection. She interpreted her results as evidence of a S-R variation, the R form being

non-toxicogenic, and suggested that under certain conditions the R form becomes the predominant type and overgrows the toxigenic organisms. The present communication is essentially a development of this thesis.

A serious objection to Cowan's hypothesis was provided by experiments previously reported by Powell (1923), who had found that with recently isolated strains of *C. diphtheriae* the property of virulence, which is an expression of toxigenicity, remained remarkably constant. Repeated single-cell isolations carried out over a period of two years yielded sub-strains which showed the same degree of virulence as the parent strain. Powell's results, frequently quoted as evidence of the stability of *C. diphtheriae*, are flatly contradicted, however, by those of Crowell (1926), who also used the method of single-cell isolation. Crowell's work seems to show conclusively that a small minority of the cells composing a virulent culture are completely non-toxicogenic. In order to reconcile these conflicting results it may be suggested that Crowell was fortunate in his chance selection of a non-toxicogenic cell. It is also possible that the strains which he studied had been maintained for a considerable time in the laboratory and were therefore in a dissociating phase: he gave no information on this point. On the balance of the evidence, and taking into account the experience of earlier workers such as Bernhardt (1915), it seems justifiable to accept the view that cultures of this organism may undergo dissociation and that non-toxicogenic variants are the result.

In more recent times the study of these intrinsic properties has to a large extent been abandoned in favour of research on the extrinsic regulators of toxin production. The constitution of the nutrient medium has received particular attention and considerable improvement in the yield of toxin has thereby been achieved. Two separate lines of investigation have been followed: the elaboration by more or less empirical methods of a medium having as its basis an infusion or digest of meat, and the search for a chemically defined medium capable of supporting growth with a high yield of toxin. Digest media prepared by the methods described by Pope and Linggood (1939) will yield toxins with a value of 70 Lf doses per ml. or more, while Mueller and Miller (1941) have obtained even better results with chemically defined media. The disadvantage of these is the high cost of the pure amino-acids which they contain and it is presumably for this reason that digest media are usually preferred for the large-scale production of toxin.

Within recent years two further communications have appeared which deal with the subject of variation in *C. diphtheriae*. In both of these, the first by Regamey (1944) and the second by d'Antona and Falchetti (1946), the authors were able to demonstrate the presence of two different types of organism in the parent P.W. 8 cultures. One of these had the characters of an organism in the R phase and was highly toxigenic, whereas the other, which resembled an S form, was a poor producer of toxin. The results of the present studies disagree with these findings on a number of points. It will be advantageous, therefore, to postpone further discussion of these papers until my own findings have been presented.

## EXPERIMENTAL

The P.W. 8 strain used throughout this investigation was that designated CN 77 in the Wellcome Physiological Research Laboratories' collection. It originated several years ago from a single colony taken from a plate culture of the original P.W. 8 strain. In the laboratory from which I write strain CN 77 has been employed for routine toxin production during the last two years, usually with good results but with variations in potency of the kind described above. At the time

when these studies were begun, the routine medium consisted of equal parts of veal infusion and Martin's pig-stomach digest, with the addition of 1.0 per cent. of sodium acetate, 0.6 per cent. of maltose and 0.2 per cent. of glucose—an unusually high content of fermentable carbohydrate. At a later stage this medium was abandoned in favour of a "straight-line" tryptic digest of ox meat prepared according to the method of Pope and Linggood. For experimental purposes these media, with the addition of phenol red as indicator, were distributed into test-tubes or 8 oz. "medical flat" bottles which were incubated in a sloping or flat position in order to maintain the optimal surface-volume ratio. When a series of tubes or bottles had to be seeded with the same culture, a uniform suspension was prepared and a constant volume equivalent to 100 million organisms added to each tube or bottle.

*Evidence that irregularity of growth and toxin formation is not due to extrinsic agents*

The following experiment will establish that intrinsic properties may determine the amount of toxin produced.

A flask of the infusion-digest medium was distributed into 12 bottles, 50 ml. to each. Each bottle was then inoculated with 0.1 ml. of a saline suspension of strain CN 77 from a 24-hour Loeffler culture. After 48 hours' incubation it was apparent that four of these cultures would fail to produce toxin: the medium had become highly acid (pH 5.5), there was no pellicle formation and only a moderate amount of deposit. The remaining 8 bottles showed normal growth at this stage and in due course they produced toxins with values between 35 and 40 Lf doses per ml. The four "failure" bottles were re-adjusted to pH 8.0 and re-inoculated with strain CN 77 as before. On further incubation two of the re-inoculated bottles produced good growth with thick pellicles, the medium remaining alkaline, as the glucose had been used up during the first 48-hour period. When the toxins were harvested on the tenth day one showed a value of 30 and the other of 20 Lf doses per ml. This type of experiment was performed several times with essentially the same result.

These findings provide a clear demonstration that the failure of some of the cultures to show normal growth and toxin formation cannot be attributed to any deficiency in the medium, since re-inoculation may be successful. The only alternative is to assume that the variable factor is in the inoculum itself. There are serious objections, however, to this assumption. As already stated, the inoculum for each bottle was a well-dispersed suspension containing 100 million living organisms. Even if the individual bacteria differ in their ability to form toxin, aliquots of a heavy suspension must contain toxigenic and non-toxigenic organisms in the same ratio; they should therefore behave in the same manner when inoculated into a series of flasks of the



same medium. The heterogeneity would become apparent only if the suspension was so diluted that the inoculum contained so few organisms that random sampling became operative. At present no wholly satisfactory explanation can be offered to meet this objection. Nevertheless, the significance of the experiment remains; a variable factor in the inoculum determines the amount of toxin produced in a suitable medium.

### *Evidence of the heterogeneity of strain CN 77*

The next step was to test the suggestion already made in this paper that strain CN 77 consisted of a mixture of organisms which differed in their toxigenic and other properties. Proof of heterogeneity was readily obtained and the separation of toxigenic and non-toxigenic sub-strains was accomplished. Two different methods were employed. In the first the usual technique of colony selection was used; in the second the best and the poorest of a batch of broth cultures were chosen as seed for a second series and so on. It was further demonstrated that a non-toxigenic strain, once isolated, did not regain its toxigenic power, whereas a highly toxigenic sub-strain continuously produced a small number of non-toxigenic variants. The following experiments are representative.

*Colony selection method.* The parent strain was plated on Neill's blood-tellurite agar (Neill, 1937). After 48 hours at 37° C. ten colonies from the same plate were transferred to Loeffler slopes and the resulting ten sub-strains tested for toxigenicity by seeding each into 5 bottles containing the same batch of medium (glucose 0.2 per cent., maltose 0.6 per cent.). The results are shown in table I.

TABLE I

*Differences in toxin production among ten sub-strains derived by colony selection from the same parent strain of C. diphtherie*

Sub-strain no.	Potency of toxins (Lf doses per ml.) after 10 days' incubation at 37° C. tested in bottle no.*				
	1	2	3	4	5
I	45	45	45	40	<5
II	40	35	35	25	<5
III	55	50	45	45	20
IV	50	40	40	30	30
V	45	40	35	<5	<5
VI	40	35	<5	<5	<5
VII	40	40	40	40	35
VIII	40	40	35	20	15
IX	<5	<5	<5	<5	<5
X	40	35	35	30	<5

\* For toxigenicity testing each sub-strain was seeded into 5 bottles.

The ten original colonies selected for this experiment differed only slightly in appearance but it was noted at the time that no. IX

## DIPHTHERIA TOXIN PRODUCTION

was flatter and a little larger than the others, and that it had a less shiny surface. Sub-strains III and IX were kept under observation for a period of nine months. They were maintained on Loeffler's medium and tests for growth and toxin production were carried out at least once a week. Throughout this period sub-strain III, tested in the manner described, showed irregularity of toxin production and sub-strain IX consistently failed to form toxin in amounts measurable by the flocculation reaction. Both produced luxuriant growth on serum agar. On Loeffler's medium sub-strain III gave a heavier growth than sub-strain IX, which grew abundantly, however, on Loeffler's medium prepared without glucose: conversely, the addition of 0.25 per cent. glucose to the serum agar appreciably depressed its growth. If the two sub-strains were plated side by side on glucose serum-agar containing phenol red the pH changes could be readily followed. Sub-strain III showed normal reversal whereas sub-strain IX remained acid and ceased to grow.

Several experiments were performed in which the fluid medium was simultaneously inoculated with both sub-strains. Such cultures always failed to show pH reversal or pellicle formation. This was interpreted as evidence that at the start the non-toxicogenic overgrew the toxigenic organisms, thereby producing sufficient acid or other metabolite to inhibit further growth. If these cultures were re-incubated after bringing the pH back to 8.0, growth sometimes started afresh and occasionally a thin film of surface growth was obtained, but no toxin.

It was known from the work of Tasman and Brandwijk (1936) that all the glucose disappeared from a medium of this kind after 48 hours' growth. Re-inoculation at this point would therefore provide the second inoculum with a medium containing maltose but no glucose, and the success of these re-inoculation experiments might depend on this fact. If this were so, it was argued that the two sub-strains might differ in their ability to metabolise glucose, and a study was accordingly made of their behaviour in media containing a range of concentrations of these two carbohydrates.

Experiments were first carried out to determine the optimal concentration of maltose alone. This was found to lie between 0.3 and 0.4 per cent. for both types of basic medium. With sub-strain III the further addition of glucose had a profound effect on toxin formation. In small amounts this sugar regularly increased the Lf value until a critical concentration of about 0.075 per cent. was reached. Above this critical value the individual culture bottles showed irregularity of growth and toxin production as in the earlier experiments, and at concentrations above 0.25 per cent. there was uniform inhibition, every culture showing a permanently acid reaction. Five similar experiments, each done in quintuplicate, were carried out and the results were essentially the same in each. The protocol of one of these experiments (table II) shows that the mean Lf values increased

TABLE II

*The effect of glucose on toxin production by C. diphtherie  
CN 77 sub-strain III*

Culture no.	Glucose concentration (g. per 100 c.c.)	pH and pellicle formation during incubation for								Toxin tested on 10th day	
		24 hours		48 hours		72 hours		96 hours		Lf doses per ml.	pH
		pH	pellicle	pH	pellicle	pH	pellicle	pH	pellicle		
A	0	7.6	±	7.4	++	7.6	++++	7.8	++++	50	9.1
		"	±	"	++	"	++++	"	++++	45	9.4
		"	±	"	++	"	++++	"	++++	45	9.3
		"	±	"	+	"	++++	"	++++	55	9.1
		"	±	"	++	"	++++	"	++++	50	9.1
B	0.012	7.5	±	7.4	++	7.6	++	7.8	++++	55	9.2
		"	±	"	++	"	++	"	++++	45	9.5
		"	±	"	++	"	++	"	++++	45	9.3
		"	±	"	++	"	++	"	++++	40	9.4
		"	±	"	++	"	++	"	++++	40	9.3
C	0.025	7.5	±	7.4	++	7.6	++++	7.8	++++	55	9.2
		"	±	"	++	"	++++	"	++++	55	"
		"	±	"	++	"	++++	"	++++	50	"
		"	±	"	++	"	++++	"	++++	55	"
		"	±	"	++	"	++++	"	++++	55	"
D	0.05	7.4	±	7.4	++	7.6	++++	7.7	++++	55	9.2
		"	±	7.2	+	"	++++	"	++++	55	9.2
		"	±	7.4	++	"	++++	"	++++	55	9.1
		"	±	7.2	+	"	++++	"	++++	65	9.2
		"	±	7.4	++	"	++++	"	++++	45	9.4
E	0.075	7.3	±	7.3	++	7.6	+++	7.7	++++	55	9.4
		"	±	"	+++	"	++++	"	++++	65	9.3
		"	±	"	+++	"	++++	"	++++	55	9.1
		"	±	"	+++	"	++++	"	++++	65	9.2
		"	±	"	+++	"	++++	"	++++	65	9.2
F	0.10	7.2	+	7.2	++	7.0	+++	7.4	+++	<5	9.5
		"	+	7.0	+	7.0	+++	7.4	+++	50	8.6
		"	+	7.0	+	Acid	+	Acid	+	5	5.4
		"	+	7.4	+++	7.6	++++	7.6	++++	40	9.2
		"	+	7.2	++	7.6	++++	7.6	++++	65	9.4
G	0.125	7.0	±	7.2	+++	7.2	+++	7.2	+++	65	9.0
		"	±	7.0	+	7.5	+++	7.6	++++	30	9.5
		"	±	7.0	+	Acid	+	Acid	+	<5	5.4
		"	±	7.4	+++	7.5	+++	7.6	++++	65	9.2
		"	±	7.4	+++	7.5	++++	7.6	++++	65	9.2
H	0.15	7.0	±	6.8	±	Acid	±	Acid	+	<5	5.6
		"	±	"	±	"	±	"	+	"	"
		"	±	"	±	"	±	"	+	"	"
		"	±	"	+	"	+	"	++	"	"
		"	±	"	+	"	+	"	++	"	"

Pellicle formation: ± indicates a very thin film, ± a slight film and + to ++++ firm pellicles of increasing thickness.

The basic medium contained 0.4 per cent. of maltose and phenol red as indicator. The final pH values were determined electrometrically.

with each increment of glucose until a maximum of 61 Lf doses per ml. was reached at 0.075 per cent., representing a 24 per cent. increase compared with the mean value of 49 Lf doses per ml. for the same medium without glucose. Yields of this order in the cultures in which toxin was produced at a slightly higher glucose concentration were offset by the other cultures of the same series in which there was a failure of toxin formation. Substantially the same results were obtained when the parent strain CN 77 was used as the seed culture but the critical concentration of glucose was lower than for sub-strain III.

The behaviour of the non-toxicogenic sub-strain IX was next studied. In the basic medium with maltose alone this strain produced a uniform turbidity which settled after 24 hours' incubation to form a flaky deposit, the pH falling from the initial value of 8.0 to 7.2. The presence of toxin could not be demonstrated by the flocculation test. After seven serial transfers in this medium a thin surface growth appeared in some of the cultures but this was immediately disintegrated by the slightest movement, being replaced by a uniform turbidity. Reversal of pH was now more marked but toxin could still not be demonstrated by flocculation. It seemed likely that the strain might revert to the characters of the parent strain if it were continuously maintained on a glucose-free medium but this expectation was not realised. After twenty more serial transfers at 48-hour intervals it still failed to produce a firm pellicle or to form toxin in amounts demonstrable by the flocculation reaction. Similar results were obtained with two other non-toxicogenic strains isolated by colony selection. It seemed justifiable therefore to regard these non-toxicogenic sub-strains as true variants with stable properties. A study of their colonial morphology described in a later section of this paper confirmed this belief.

*Culture selection method.* The second method of demonstrating heterogeneity was to maintain the parent strain in a medium of high glucose content, choosing for each transfer the best and the poorest growth from the series of tubes inoculated. This procedure and its results are illustrated by the following experiment.

Strain CN 77 was seeded into five tubes of the infusion-digest medium containing 0.6 per cent. maltose and 0.2 per cent. glucose. After 48 hours' incubation two of the five cultures had reverted to an alkaline reaction and showed pellicles, while the remaining three cultures were strongly acid and growth was limited to a deposit at the bottom of the tube. The first culture to show pH reversal was chosen as the seed for a second series of five tubes of the same medium. Again the best of these cultures was used as seed for a third series and so on through 15 such sub-cultivations (table III).

The final series of subcultures (no. XV) was made in 50 ml. of medium so that the toxins could be assayed by flocculation. The potency of the toxins, harvested on the tenth day of incubation,

showed little variability, ranging between 40 and 46 Lf doses per ml. By this simple procedure therefore it was possible to reduce considerably the variability in toxin formation but the improvement

TABLE III

*Incidence of cultures with growth characters associated with toxin production after serial subculture of selected tubes*

Serial no. of subcultures	No. of cultures showing normal growth
I	2 out of 5
II	4 "
III	5 "
IV	3 "
V	5 "
VI	5 "
VII	4 "
VIII-XIV	5 "
XV	10 " 10

was not permanent, for after being maintained on Loeffler medium for a few weeks the strain again showed irregularity of growth and toxin formation when cultured in media with a high glucose content.

The second part of this experiment was concerned with the later behaviour of the cultures that failed to show *pH* reversal. Continued incubation resulted in death of the organisms, subcultures seldom being successful after 72 hours at 37° C. The strains could be maintained, however, by daily passage in the medium with glucose and in this they continued to produce a persistent acidity without pellicle formation. In the medium without glucose they eventually formed a readily dispersed surface growth similar to that produced by the non-toxicogenic sub-strain IX.

It thus appeared that stable non-toxicogenic variants could be obtained from strain CN 77 either by colony selection or by using media with a high glucose content. Once the non-toxicogenic variant had been temporarily eliminated the normal toxicogenic organisms would accept the high concentration of glucose presumably because the rate at which they broke down the sugar was slower than that of the non-toxicogenic variant.

#### COLONIAL MORPHOLOGY

Throughout the investigation the parent strain and its sub-strains were plated at frequent intervals. The media used were blood-agar, serum-agar, and Neill's tellurite-agar, all prepared from horse blood. The differences observed were most plainly revealed by the tellurite medium and the descriptions that follow are based on these.

The parent strain CN 77 after 24 hours at 37° C. appeared as small (0.1-0.3 mm.) convex black colonies with a shiny surface and a narrow

transparent flattened peripheral zone. After 48 hours these colonies increased to 1-3 mm. but were otherwise unchanged. At this time, however, a few colonies were noted which were grey-black in colour and flatter than the rest, with a less shiny surface and a rounded or bevelled edge but without a transparent periphery. On further incubation these colonies developed a matt surface with radial and concentric striations. Usually the centre of the colony was raised above the level of the peripheral portion; rarely it was crater-shaped. Although these two types of colony were readily differentiated it must be emphasised that colonies with an intermediate appearance were sometimes present, so that there was a gradation rather than an abrupt transition from the predominant shiny raised colony to the rare flat matt type. A total of 30 sub-strains, including the 10 already described (table I), were obtained from the original strain CN 77 and each of these was replated at intervals and tested for toxigenicity. The knowledge gained from the examination of several hundred such cultures may be summarised by the statement that sub-strain III and other toxigenic strains resembled the parent strain, whereas sub-strain IX and other non-toxigenic strains produced only the flat grey-black matt type of colony. Both Regamey and d'Antona and Falchetti described an extremely rough type of colony with crenated edge and a rugose surface. This type was never encountered in the present studies, in which all sub-strains cultured on Loeffler's medium produced a growth which was light cream in colour; pigmentation was not observed even after the cultures had been stored for many weeks either in the dark or in subdued daylight.

None of these strains fermented starch. Tests for hæmolytic activity, carried out by the method described by Hewitt (1947), showed that the parent strain and its toxigenic sub-strains were unable to hæmolyse horse or rabbit red cells but the non-toxigenic sub-strain IX produced rapid hæmolysis of both. This unexpected finding emphasises still further the extent to which this strain differs from the original.

solely on the ability of the organism to produce toxin in the animal body.\* The findings emphasise a limitation of the virulence test as ordinarily performed; it gives no indication of the amount of toxin which the strain is capable of producing under optimal conditions. In order to measure these differences it was necessary to make repeated determinations of the M.L.D. and of the Lf or Lr dose of cultures in a suitable medium. The results of one such experiment are given in table IV. Strain no. 3, included for comparison, was a

TABLE IV  
*Toxin production by sub-strains III and IX, and by a  
mitis strain (no. 3)*

Strain	Toxin content expressed as the Lr/500 dose									
	Days									
	1	2	3	4	5	6	7	8	9	10
Sub-strain III	0.0025	0.00003 (27)	0.000036 (40)	0.000072 (40)	0.000072 (41)	0.000062 (46)	0.000014 (40)	0.00001 (53)	0.00001 (47)	0.00001 (42)
Sub-strain IX	0.018	0.018	0.003	0.01	0.0075	0.007	0.005	0.003	0.007	0.003
<i>Mitis</i> no. 3	0.056	0.0016	0.0019	0.002	0.001	0.001	0.001	0.001	0.001	0.001

The figures in parenthesis are the Lf values (no. of Lf doses per ml.) of the toxins produced by sub-strain III.

virulent *mitis* type which had been recently isolated. Each strain was seeded into 50 tubes of the same batch of medium, and five cultures of each strain were removed every day from the incubator and tested separately for toxin content. The figures shown in table IV are the means of each set of 5 determinations.

In view of these findings it is obviously incorrect to speak of sub-strain IX and similar variants as non-toxicogenic strains. Instead of possessing the abnormally high in-vitro toxicogenicity of a P.W. 8 strain they are comparable in this respect to the majority of virulent strains isolated from throat swabs. In this connection it will be remembered that the M.L.D. for man is not much larger than that for the guinea-pig.

#### PRACTICAL APPLICATIONS

As a result of these studies a number of modifications were introduced into the routine method of large-scale production. These changes, including those affecting the culture medium, brought about an improvement in the yield of toxin and a brief description of our current practice may therefore be of interest to other workers in this field.

The highly toxicogenic sub-strain III replaced the parent P.W. 8 strain, from which it was derived by colony selection. Sub-strain III

\* The evidence on which this statement is based will be given in a later publication.

is maintained continuously on tryptic digest medium (Pope and Linggood) containing maltose 0.3 per cent. and glucose 0.05 per cent. with phenol red as indicator. This medium is adjusted to contain 100  $\mu$ g. of ferrous iron per litre. Subcultures are made at intervals of 48 hours, never longer. On each occasion five tubes of the medium are inoculated and the one which shows the earliest reversal of pH and the firmest pellicle is chosen as the seed for the succeeding five subcultures. A fluid medium has two advantages: any change from the normal appearance of the pellicle is an indication that dissociation is taking place; and the presence of a contaminant is usually obvious at a glance. However, if it is preferred to maintain the strain on a solid medium, plain inspissated serum or Loeffler's medium made from glucose-free broth should be employed. Loeffler's medium as ordinarily prepared contains 0.25 per cent. of glucose plus the sugar that was present in the serum. It is an advantage to incorporate an indicator such as phenol red in solid media so that the reaction of the culture may be evident.

The same tryptic digest medium with glucose and maltose is used for the actual production of toxin. The optimal period of incubation before harvesting has not yet been fixed. Under experimental conditions the toxin-production curve reaches its maximum on the fifth or sixth day. Under the conditions of large-scale production, however, a small proportion of the flasks show a lag in toxin production and in these the maximum may not be reached until the tenth day. Thus the mean potency of the batch may be increased by longer incubation but this advantage is off-set by the greater concentration of metabolites and products of bacterial autolysis which accumulate in the medium. The presence of these substances is undesirable whether the toxin is to be used in the preparation of diphtheria prophylactics or for hyper-immunisation of horses. The relative merits of early and late harvesting of the toxin cannot be finally judged until further information becomes available.

#### DISCUSSION

The facts established by these experiments are in harmony with the present concept of variation which regards a bacterial strain, even one derived from a single cell, as a heterogeneous population whose individual cells are potentially or actually endowed with different properties, though the fundamental characters of the species are shared by all. Some organisms readily dissociate so that this heterogeneity is easily demonstrated, whereas in others it is revealed only by colony selection or single-cell isolation, or by altering the cultural environment of the strain as a whole so that a type of cell which was previously in the minority becomes the dominant cell type. This concept, first clearly enunciated by Bernhardt (1915), has received considerable support from the more recent work of Lewis (1934) on



*Bacterium coli-mutabile* and of Deskowitz (1937) on *Salmonella aertrycke*. To these examples, and many others not cited, it is now possible to add the heterogeneity of the present strain of *C. diphtheriae* in respect of its carbohydrate metabolism and toxigenic properties. Whether other strains behave in a similar manner is not yet known but preliminary experiments suggest that the toxigenic power of a recently isolated strain can be increased by selective cultivation.

It is now necessary to compare these results and the deductions made from them with the previous studies of Regamey and of d'Antona and Falchetti to which brief reference has already been made. Regamey studied four P.W. 8 strains named "London," "Basel," "Toronto" and "Dresden" after the cities from which they were obtained. Each of these when cultured on Loeffler's medium gave rise to two types of colony. Variant "A" was yellow-white in colour, hemispherical and small; variant "B" was markedly chromogenic ("couleur chamois"), flattened and considerably larger than A. When grown in suitable media variant A produced no detectable toxin and failed to show pH reversal, whereas B produced toxins with a potency of 40 Lf doses per ml. With strain "London" these variants became stable after a number of subcultures. It will be noted that the present findings disagree with those of Regamey on two important points: my sub-strains failed to show differences in chromogenicity, and the property of toxigenicity was associated with the small shiny convex colony and not with the large flat variant.

d'Antona and Falchetti also sought to explain irregularity of toxin production in terms of bacterial variation. Working with a P.W. 8 strain, they found that cultures on plain agar or inspissated serum showed little evidence of differentiation, but when rabbit blood-agar was used two distinct types of colony were obtained, which they illustrated and described in detail. One type showed all the characters of an organism in the smooth (S) phase: 1-2 mm. in diameter, circular, convex and shiny, with an entire edge, an opaque centre and a transparent periphery. A stable suspension was obtained by emulsifying the growth in saline. In contrast to this, the second type was typically rough, flat and grey-white in colour, with an irregular edge and a rough surface which did not reflect the light. The growth was dry and friable and was suspended with difficulty in saline. After several days' incubation the colonies became even flatter and increased in size to a diameter of 5 mm. In stained films the S forms showed long Gram-negative rods completely free of polar granules, whereas the R forms consisted of short Gram-positive rods which showed marked bipolar staining. In the medium normally used for toxin production the S form produced uniform turbidity and failed to show either pH reversal or pellicle formation. The R form, on the other hand, produced a typical pellicle with normal pH reversal. Toxin formation was minimal with the S form (5 Lf doses per ml.), whereas the R form yielded toxins with a value of 40 Lf doses per ml.

When the two sub-strains were tested for virulence in guinea-pigs no appreciable difference could be demonstrated. From this finding these workers concluded that the S form was virulent but not toxigenic whereas the R form was both virulent and toxigenic. The results of my own virulence tests thus agree with those of d'Antona and Falchetti; but while they postulate the presence of a virulence component distinct from toxigenicity, I consider that the results can be explained on a quantitative basis with toxin formation as the sole lethal factor. Continuing their researches, these authors found that neither form retained its characters on further subcultivation but that either form gradually changed into the other, and all stages of transition from one to the other were recognised. This change from R to S is a most unusual finding in studies of bacterial variation. It does not tally with my experience, which indicated that toxigenic gave rise to non-toxigenic organisms, but never the reverse. The most serious disagreement, however, concerns the colonial morphology of the toxigenic strains. They found that toxigenicity was a property of the rough variant, whereas in my experience it is the smooth colony which produces toxin and the flat lustreless type of colony that is deficient in this property. Colonies with the degree of roughness described and illustrated in their communication were never observed in the present investigation. All the sub-strains which I studied, whether toxigenic or not, would probably be regarded by d'Antona and Falchetti as examples of their intermediate S-R forms. There is general agreement, however, on what is after all the most important finding: the modern P.W. 8 strains are in a highly unstable condition and will dissociate into a phase of low toxigenicity, this change being associated with an alteration in the ability to metabolise carbohydrates. So long as this phenomenon is recognised the colonial appearances assumed by the organism at any particular stage of its dissociation are more of academic than practical importance. The P.W. 8 strain has been in existence for half a century; it is not surprising therefore that sub-strains from different laboratories should now show marked cultural differences.

One further point deserves mention because of its possible significance in clinical practice. Studies of the blood-sugar in clinical practice reveal that there is often hypoglycæmia in diphtheria. The administration of glucose either intravenously or by mouth is therefore a logical procedure and is recommended as a useful adjuvant to specific treatment with antitoxin (Harries and Mitman, 1947). The usual explanation is that glucose helps to protect the heart from the action of the toxin. If the results of this investigation can be applied to other strains of *C. diphtheriæ* an alternative explanation may be suggested. By increasing the glucose concentration in the blood and tissues the organisms in the tonsils or other sites of infection will be brought into contact with a medium which is unfavourable for toxin production. A blood-sugar level of 0.15 per cent. can be

easily maintained by administration of glucose and my in-vitro experiments show that at this concentration the P.W. 8 strain may cease to form toxin. Experiments are being carried out to test the validity of this hypothesis.

### SUMMARY

1. A study was made of the causes of irregularity in toxin formation by a Park-Williams no. 8 strain of *C. diphtheriae*.

2. The strain investigated was found to be composed of a mixture of highly toxigenic organisms and others with a much reduced capacity to form toxin. The two types of organism were separated. Besides their difference in toxigenicity they were found to differ in colonial morphology and in their reaction to glucose in the culture medium.

3. The presence of glucose above a concentration of 0.075 per cent. favoured the dissociation of the toxigenic sub-strain, with the production of the poorly toxigenic variant. This, after first isolation, appeared to be stable.

4. Regularity of toxin formation was thus found to depend upon the use of a strain which was in the highly toxigenic phase. Methods of isolating such strains and of maintaining them in this condition are described.

5. These findings are compared with the results recently published by other workers in this field.

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(*Shigella shigæ*)

## REDISTRIBUTION OF BODY FLUIDS IN DYSENTERIC TOXAEMIA

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(PLATE CII)

CONSIDERABLE experimental evidence has accumulated to show that a wide variety of factors cause alterations in the volume and distribution of body fluids. It has been claimed that intravenous injection of certain bacterial toxins produces blood-volume changes in experimental animals. Yannet and Darrow (1933), using diphtheria toxin, and Penner and Bernheim (1942), using dysentery (shiga) toxin, demonstrated hæmoconcentration, their evidence being based on the red-cell count, the hæmoglobin percentage and the hæmatocrit reading. No direct estimations of blood volume were made by these workers.

I have undertaken a study of the blood-volume changes in rabbits given dysentery (shiga) toxin intravenously, using direct measurement as well as the above indirect criteria. Plasma proteins, extra-cellular fluid and the behaviour of Evans blue given intravenously were also studied in an endeavour to explain these changes. Autopsy and microscopic examination helped to trace the distribution of body fluids.

### MATERIAL AND METHODS

Healthy adult rabbits weighing 2.3 kg. were used. The animals were not anaesthetised. Injections were given into and blood collected from the marginal vein of the ear. As rabbits are known to possess a readily available store of fluid for replacing fluid loss (Cameron and Courtice, 1946-47) they were kept on a dry diet for two days and fasted for 24 hours before toxin was injected. Controls were treated similarly.

The toxin, provided in generous amounts by Dr C. L. Oakley of the Wellcome Physiological Laboratories, Beckenham, Kent, contained 25 units per c.c. By trial, it was found that 0.25 unit killed a rabbit, on an average, in 60-72 hours and this dose was adopted. The toxin was diluted in physiological saline so that the 0.25 unit was contained in 1 c.c. (i.e. 1 in 100). As controls, rabbits under identical conditions of caging and feeding were given toxin which had been heated at 75° C. for 15 minutes.

*Hematological examinations*

*Red blood corpuscles.* Total red cells were counted with the Thoma-Zeiss hæmocytoimeter. The hæmatocrit method was used for estimating corpuscular volume. Capillary glass tubing about 0.8 mm. bore was selected for this purpose by running a length of mercury into the lumen and measuring it at intervals. Any tube with an uneven bore was discarded. The hæmatocrit tube was spun for 45 minutes at 3000 r.p.m. I am indebted to Dr F. C. Courtice, Department of Physiology, Oxford, for details of the method.

*Plasma volume* was estimated with Evans blue according to the method of Courtice (1943-44). The strength of dye solution used was 60 mg. per 100 c.c. in physiological saline and the dose for a rabbit was 2 c.c. Dye concentrations were estimated in a photo-cell colorimeter. When blood is withdrawn a few minutes after the injection of the dye, the error caused by the small amount of dye which has disappeared up to the time of withdrawal may be corrected by estimating the dye concentration at 9, 15 and 30 minutes and extrapolating the curve so obtained back to zero. This theoretical value represents the concentration of the dye if it were completely mixed at the moment of injection. However, the taking of three samples of blood for each estimation was impracticable in rabbits which had to be bled several times a day for the various examinations. Single readings were therefore made 6 minutes after injection of the dye. This was found by trial and from the experience of Courtice to be 94 per cent. of the zero reading. The necessary corrections were made accordingly.

*Disappearance curves of Evans blue.* In order to study changes in capillary permeability, disappearance curves of Evans blue were plotted before and after injection of toxin. Hopps and Lewis (1947) used Evans blue for a similar study. This dye is firmly bound to the plasma proteins (Courtice) and behaves like serum albumin as far as permeability of membranes is concerned (Rawson, 1942-43). In order to trace the path and the distribution of fluid leaving the blood vessels, 4 c.c. of a 2 per cent. solution of Evans blue were injected into rabbits which had received toxin, and into controls. The animals were killed forty-five minutes later and the distribution of the dye in the tissues studied.

*Extra-cellular fluid.* This was estimated with sodium thiocyanate according to the method of Crandall and Anderson (1934). Rabbits were given 20 mg. per kg. of sodium thiocyanate as a 2.5 per cent. solution. The plasma proteins were precipitated with 20 per cent. trichloroacetic acid. Ferric nitrate was added to the filtrate and the colour, developed rapidly, matched in a photo-cell colorimeter. By so timing the injections of Evans blue and thiocyanate it was possible to use one sample of blood for the estimation of both plasma volume and extra-cellular fluid.

*Plasma proteins and non-protein nitrogen.* These were estimated by micro-Kjeldahl digestion and titration according to the method of Rimington and Bickford (1947), 0.1 c.c. of plasma being used for each of the protein fractions. For incineration, 50 per cent. (w/v) sulphuric acid containing 1 per cent. selenium dioxide was used. A drop of saturated copper sulphate was added to each 2 c.c. In distilling, 50 per cent. (w/v) sodium hydroxide was used for alkalinisation. The distillate was collected into saturated boric acid. The ammonia was titrated directly with sulphuric acid, using methyl red as indicator. The factor 6.25 was used to convert nitrogen to protein values.

A record was kept of the clinical condition of the animal, autopsies were performed on all and sections prepared of the chief organs.

## RESULTS

For about 2 hours after the injection of toxin the animals appeared normal. The first change noticed was the occurrence of irregular periods of rapid breathing. In about 4 hours rabbits appeared to be

less active and their coats became rough. About this time in some but a little later in others, the ears were drooping, the extremities cold and bleeding was slow and difficult. These signs gradually increased until in 24 hours the animals appeared very ill with the abdomen noticeably distended. From this time onwards a gradually increasing and spreading paralysis appeared, sometimes starting in

TABLE I

*Effect of the intravenous injection of 0.25 unit of dysentery toxin  
(average of 6 experiments)*

Time	Hb. (per cent.)	R.B.C. (millions)	Hæmato- crit (per cent.)	Plasma vol. (c.c.)	Red cell vol. (c.c.)	Extra- cellular fluid (c.c.)	Proteins (g. per 100 c.c.)			N.P.N. (mg. per 100 c.c.)	Weight (g.)
							Total	Albumin	Globulin		
Before	103.0	6.69	42.4	87.41	64.90	588.3	7.263	6.050	1.213	18.33	2350
2 hrs. after	105.5	6.78	42.6	...	...	...	...	...	...	...	...
4 hrs. after	108.3	6.83	41.9	79.01	57.23	...	6.990	5.780	1.210	96.66	...
24 hrs. after	106.7	7.00	41.3	75.15	52.5	616.9	7.084	5.718	1.366	136.60	2292
48 hrs. after	97.0	5.87	38.6	72.30	45.5	728.6	6.649	5.350	1.299	180.00	2280

TABLE II

*Effect of intravenous injection of 0.25 unit of heated dysentery toxin  
(average of 4 experiments)*

Time	Hb. (per cent.)	R.B.C. (millions)	Hæmato- crit (per cent.)	Plasma vol. (c.c.)	Red cell vol. (c.c.)	Extra- cellular fluid (c.c.)	Proteins (g. per 100 c.c.)			N.P.N. (mg. per 100 c.c.)	Weight (g.)
							Total	Albumin	Globulin		
Before	100.2	6.52	40.92	99.20	68.95	527.6	6.594	5.037	1.557	15.0	2288
2 hrs. after	99.5	6.47	39.72	...	...	...	...	...	...	...	...
4 hrs. after	94.5	6.00	37.97	99.72	61.40	...	6.609	5.218	1.391	17.5	...
24 hrs. after	88.0	5.42	33.30	103.60	52.00	534.6	6.703	5.359	1.344	22.5	2300
48 hrs. after	78.7	4.87	30.4	107.95	47.55	529.4	6.704	5.407	1.297	22.5	2263

the fore limbs but more often in the hind limbs. By 48 hours the animals lay stretched out in the cage, completely flaccid. The extremities and ears were cold, the abdomen distended and the veins collapsed, so that bleeding became very difficult. In two of the rabbits blood had to be collected by cardiac puncture. Coma soon supervened and deepened till they died in 60-72 hours. From 24 hours they had been completely off food. In spite of starvation and severe illness the loss of weight was not marked, averaging about 70 g. in 48 hours.

The controls remained quite well. They, too, lost weight—about 25 g., probably due to the repeated bleeding and the operative procedures to which they had been subjected.

The findings in all the animals were similar. Tables I and II show the means of six experiments in which toxin was injected intravenously and of a control group of four experiments where heated toxin was given.

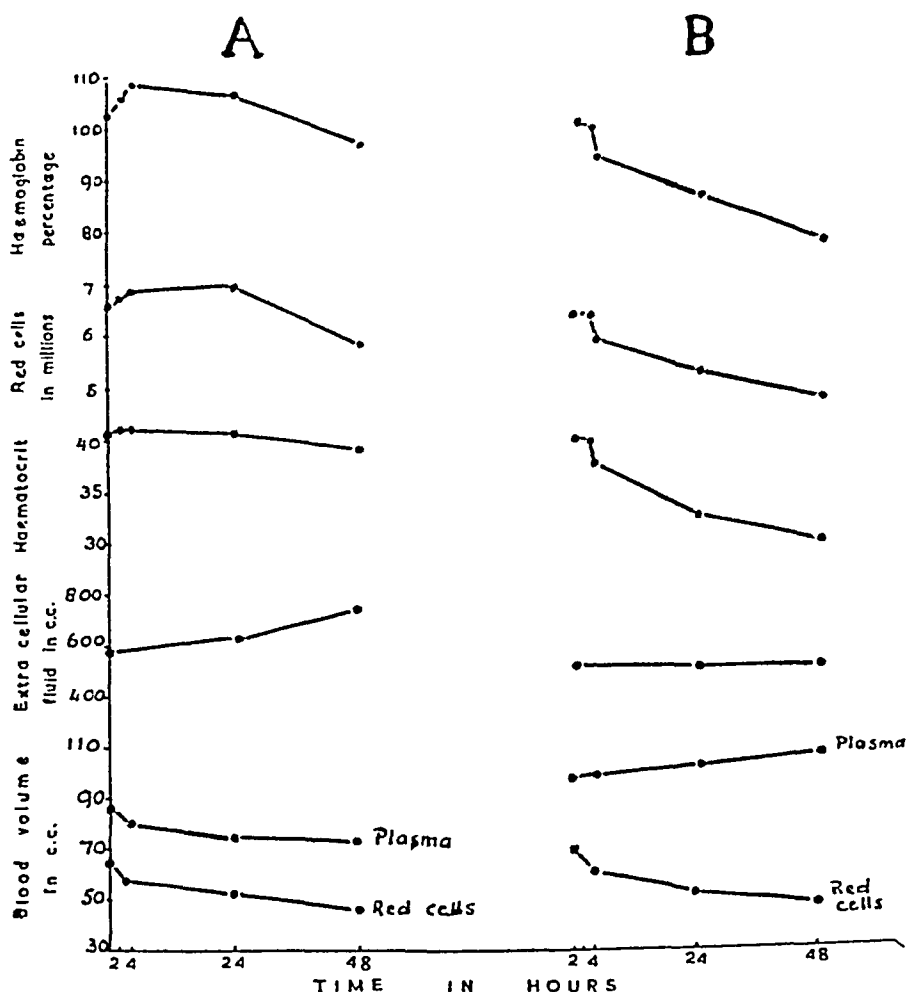


FIG. 1.—Effect of intravenous injection of dysentery toxin on blood volume and extra-cellular fluid. A. Toxic group (received 0.25 unit of toxin each). B. Control group (received 0.25 unit of toxin heated at 75° C. for 15 minutes).

1. The hæmoglobin percentages, red cell counts and hæmatocrit readings (fig. 1) ran more or less parallel and began to show increases as early as 2 hours after the injection of toxin, reaching a maximum in 4-6 hours. This level was maintained for 24 hours, after which a slow decline set in. At first sight the increase appears small and

insignificant, but when a small animal like the rabbit is repeatedly bled at short intervals it loses an appreciable percentage of its blood. The plasma is rapidly replaced but the red cells are not so rapidly regenerated. This is clearly shown in the controls, a progressive

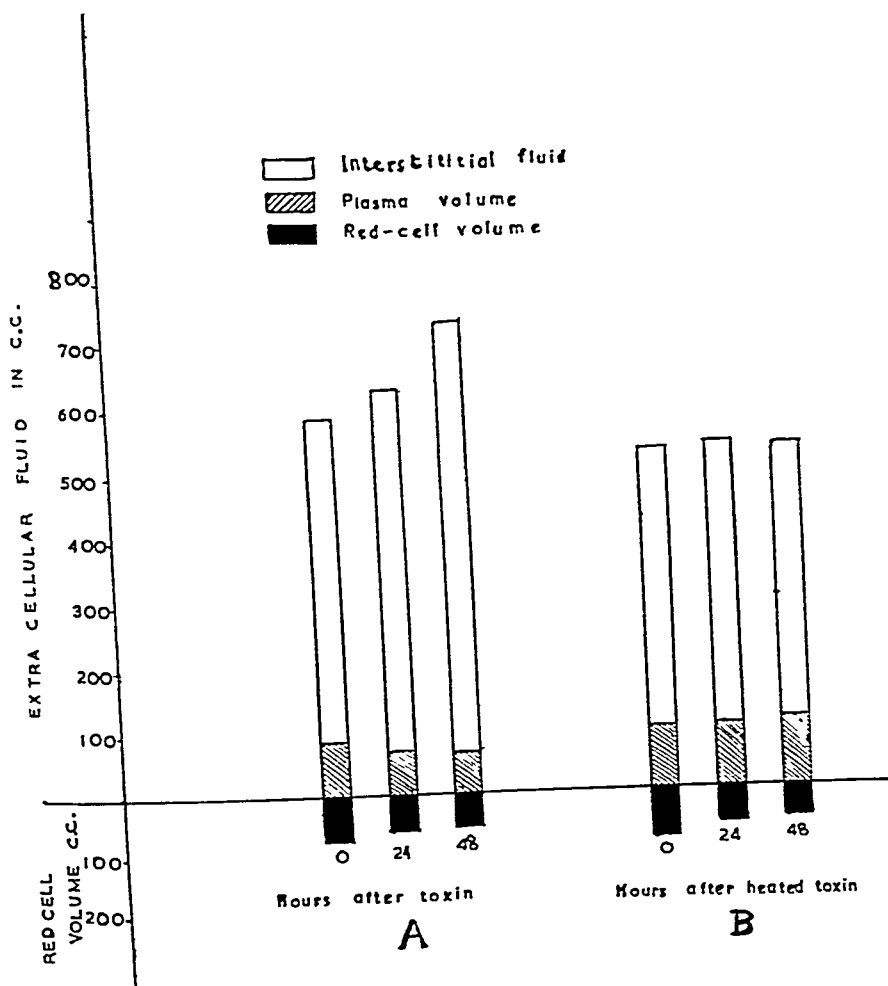


FIG. 2.—Behaviour of extra-cellular fluid after intravenous injection of 0.25 unit of dysentery toxin. A. Toxic group (average of six experiments). B. Control group (given the same dose of toxin heated at 75° C. for 15 minutes—average of four experiments).

fall in all the readings indicating uncomplicated hæmorrhage. In these experiments each rabbit was bled about 25 c.c. in 24 hours. It therefore lost 10-12 c.c. of red cells or 15-20 per cent. of its total red-cell content. The corrected degree of hæmoconcentration would thus be 15-20 per cent. higher than the findings shown.

2. A decrease in plasma volume was noticeable in 4 hours in animals receiving toxin. In 24 hours it was marked (fig. 1). On an



average about 13 c.c. of fluid were lost, or one-seventh of the total circulating plasma. A further smaller fall occurred in the next 24 hours. In the control group there was no reduction in plasma volume, any fluid lost in bleeding being rapidly replaced. Indeed a slight increase was found in most animals, probably a reaction to hæmorrhage.

3. The total volume of circulating red cells was progressively reduced in both toxic and control groups by the bleeding to which the animals were subjected.

4. The volume of extra-cellular fluid, which is made up of plasma and interstitial fluid, remained unchanged in the controls but was increased in the toxic animals. Fig. 2 shows the relation of extra-cellular fluid and blood in toxic animals and controls. In view of the fact that the volume of plasma was reduced, the increase in extra-cellular fluid must have been due entirely to an increase in the interstitial fluid.

5. The percentage of proteins in the plasma showed no appreciable change after injection of toxin. Yannet and Darrow obtained a similar result with diphtheria toxin and, as the percentage remained unchanged while the volume of plasma was reduced, they inferred that the total quantity of circulating proteins must have been reduced. Having estimated the plasma volume in my experiments it was possible to calculate the total circulating proteins, as shown in table III. A progressive fall from 6.348 to 4.808 g. is seen, i.e. a

TABLE III

*Effect of intravenous injection of dysentery toxin on plasma proteins*

		Before Injection	4 hrs. after	24 hrs. after	48 hrs. after
<b>Toxic group</b> 0.25 unit of toxin intra- venously (average of 6 experiments)	Total protein (g.)	6.348	5.522	5.310	4.808
	Albumin (g.)	5.288	4.566	4.200	3.968
	Globulin (g.)	1.060	0.956	1.020	0.840
<b>Control group</b> Heated toxin intra- venously (average of 4 experiments)	Total protein	6.537	6.500	6.940	7.240
	Albumin	4.990	5.204	5.583	5.826
	Globulin	1.547	1.386	1.357	1.414

reduction of about 20 per cent. This fall suggests that a fluid rich in proteins was passing out of the blood vessels. It will be noticed that the fall in proteins is confined almost entirely to the smaller albumin molecule.

6. In 4 hours a definite increase was found in the non-protein nitrogen. It continued to rise for 24-48 hours, when figures of 150-200 mg. per 100 c.c. were reached. Thereafter it declined, and, in the only rabbit that survived, returned to normal in 5 days. The study

of this nitrogen retention is outside the scope of the present investigation, but the absence of histological changes in the kidneys and the rapid recovery suggest the operation of extra-renal factors such as low filtration pressure and tissue destruction.

7. When Evans blue was injected 48 hours after the toxin the concentration of the dye in the blood was higher owing to the reduction in plasma volume, but the rate of disappearance was the same as before the toxin (fig. 3). Either the capillaries had recovered from

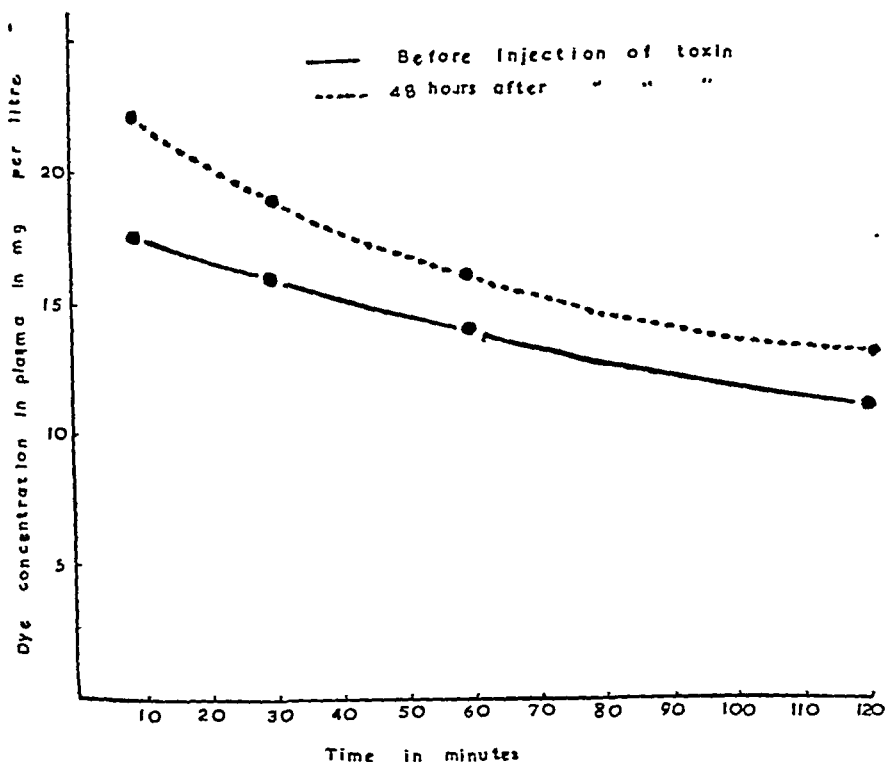


FIG. 3.—Disappearance curves of Evans blue before and 48 hours after injection of dysentery toxin.

the effects of the toxin and ceased to leak or dye and fluid were leaving the capillaries in the same proportions as they existed in the blood. To clarify this point the dye was injected 16 hours after the toxin, when the effect on the capillaries was expected to be at its maximum. The result was the same (fig. 4). The dye concentration was higher but the rate of disappearance ran parallel to that obtained before injection of toxin. It is reasonable to conclude from this that fluid with a protein content similar to plasma was leaving the blood.

8. Antitoxin injected half an hour after toxin protected rabbits and prevented the blood changes. Partial protection was afforded

when the interval was 1 hour, but after 2 hours antitoxin had no effect, the animals dying in the usual time with all the blood changes seen in the untreated ones.

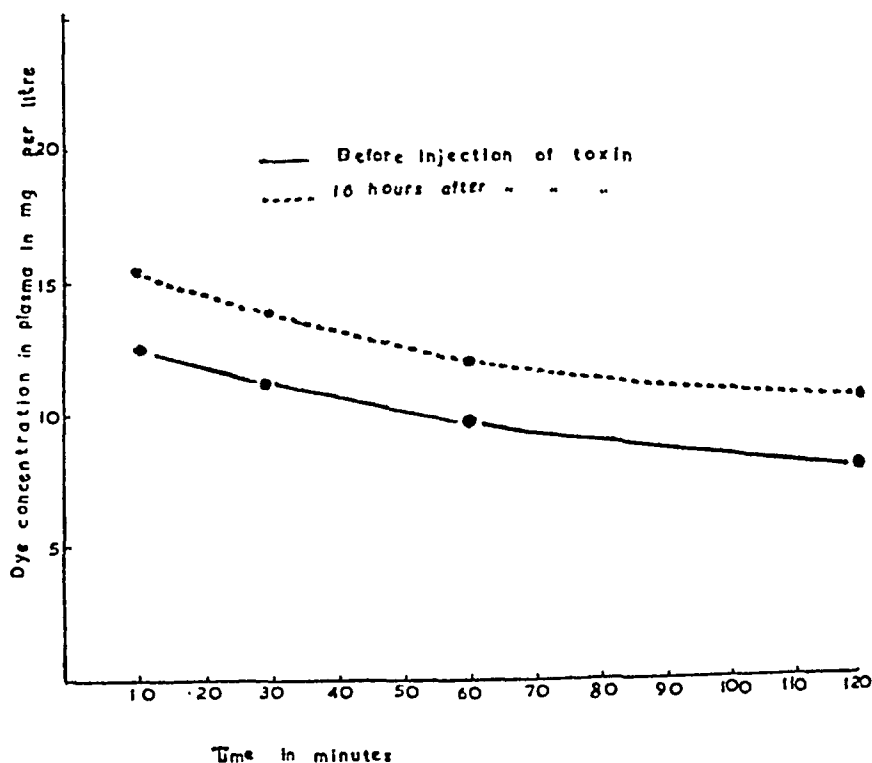


FIG. 4.—Disappearance curves of Evans blue before and 16 hours after injection of dysentery toxin.

#### *Macroscopic appearances after shiga toxin*

The skin, subcutaneous tissues, muscles of the abdomen, thorax and limbs appeared normal and free from œdema. The retroperitoneal tissues showed definite but not excessive œdema. In one case a small amount of clear straw-coloured ascitic fluid was found.

The heart contained dark fluid blood. The lungs were congested but not œdematous. There was no fluid in the pleural cavities. The liver, spleen and kidneys were engorged, the adrenals and stomach normal. The distal part of the ileum was thick and œdematous, the cœcum swollen and heavy, due to gross œdema which extended to the proximal colon. The wall of the cœcum was very thick, contrasting strikingly with its thin transparent structure in the normal rabbit. Its mucous membrane was œdematous and congested, and dotted with numerous petechiæ which ran together in the proximal part, producing large areas of hæmorrhage. The distal ileum and proximal colon showed similar but much milder changes. In animals

which survived for 72 hours superficial erosion of the mucosa had developed, producing the beginnings of the familiar dysenteric ulceration. Tables IV and V show the weight of the cæcum in toxic

TABLE IV

*Weight of organs (g.) in rabbits 48-54 hours after intravenous injection of 0.25 unit of dysentery toxin*

Rabbit	Total weight	Cæcum	Liver	Kidney	Lungs	Heart
18 . . .	2200	93.5	101	20.5	13	8.5
19 . . .	2025	57	85	19	11	7.5
20 . . .	2125	72	97	20	12	9
27 . . .	2700	69	90	23	13	10
28 . . .	1750	74	102	22	9	9
31 . . .	2750	69	120	29	19	13
35 . . .	2500	57	110	24	16	12
Average . . .	2300	70.5	99.3	22.5	13.3	9.9
Weight per kg. of rabbit	1000	30.6	43.1	9.8	5.7	4.3

TABLE V

*Weight of organs (g.) in control rabbits 48-54 hours after intravenous injection of 0.25 unit of toxin heated at 75° C. for 15 minutes*

Rabbit	Total weight	Cæcum	Liver	Kidney	Lungs	Heart
1 . . .	2880	51	108	18.9	14	10
2 . . .	2150	47	94	19	11	9
23 . . .	2700	59.3	113	22	14.5	12
29 . . .	2400	49	104	21	13	11
Average . . .	2532	51.6	104	20.2	13.1	10.3
Weight per kg. of rabbit	1000	20.37	41.6	8.1	5.2	4.1

animals and controls, with the weight of some of the other organs for comparison. The average weight of the cæcum was 51.6 g. in the controls and 70.5 g. in the toxic group, or 20.37 and 30.6 g. per kg. of rabbit respectively, which means an increase in weight of 50 per cent. None of the other organs showed any appreciable difference in the two groups.

When Evans blue was injected before killing the animals the difference in the two groups was striking. In the toxic group there was diffuse blue staining of the wall of the cæcum and adjacent ileum and colon. There was also faint staining of the serous coat of the other abdominal organs. The control group showed only a blue tinge in the peritoneal coat of the abdominal viscera. Since the

diffusion of Evans blue may be taken as an index of permeability of the capillaries to proteins, the conclusion is that fluid containing protein had leaked out of the capillaries and accumulated in the stained areas.

### *Microscopic appearances*

The *cæcum* shows extensive œdema of all its coats (figs. 5-7), the submucosa being particularly affected. Blood vessels and lymphatics are dilated and scattered hæmorrhages are present in the mucous and submucous layers. In the earlier stages there is complete absence of inflammatory cells, but in animals surviving for 72 hours superficial erosion of the mucosa with some cellular reaction, possibly due to secondary infection, is seen. The *lower ileum* and *proximal colon* show similar but much less severe lesions. The *liver* is congested and shows cloudy swelling or early fatty change. The *spleen* is engorged. The *kidneys* show cloudy swelling and congestion. The *lungs* are congested, with thick alveolar septa containing tortuous dilated capillaries.

### DISCUSSION

A reduction in blood volume is a constant result of the intravenous injection of dysentery (shiga) toxin in rabbits, the indirect evidence of hæmoconcentration being supported by direct measurement of blood volume. The occurrence of hæmoconcentration indicates an actual loss of fluid from the blood. In the absence of diarrhœa or other external loss, the fluid which leaves the blood must collect elsewhere in the body, and experience suggests that in dysentery the wall of the large intestine is the main depot where this occurs. Of the many factors known to cause movements of body fluids, *e.g.* organic and inorganic poisons, bacterial toxins and burns, the majority act by altering capillary permeability. Shiga toxin appears to be no exception, and the increased permeability of the capillaries is responsible for the diffusion outwards of protein-rich fluid resembling plasma.

The path taken by the fluid leaving the capillaries and its ultimate fate are shown by disturbances in the distribution of extra-cellular fluid. Normally about 20 per cent. of the total body fluid is extra-cellular, 5 per cent. being plasma and 15 per cent. interstitial fluid. The increase of interstitial fluid in the toxic animals indicates where fluid leaving the blood might be accommodated. No doubt other sources may contribute to this increase, such as water liberated by tissue breakdown, and certainly the rise in non-protein nitrogen in the absence of structural kidney damage supports such a view. But the plasma diffusing from the capillaries must play an important part in the increase, for it also remains within the body. The passage of Evans blue from the blood along with fluid and proteins into the œdematous areas allows of the identification of such regions. Although

BODY FLUIDS IN DYSENTERIC TOXÆMIA



FIG. 5.—Edema of caecal wall 48 hours after injection of dysentery toxin. Hæmatoxylin and eosin.  $\times 50$ .



FIG. 6.—Edema of mucosa and submucosa of caecum of rabbit, with hæmorrhage into mucosa, 60 hours after injection of dysentery toxin. Hæmatoxylin and eosin.  $\times 50$ .



FIG. 7.—Normal caecum of rabbit. Hæmatoxylin and eosin.  $\times 50$ .



the œdema is most obvious in the cæcum, it nevertheless constitutes only a small part of the total increase of interstitial fluid. The cæcum increased in weight by about 19 g., a small fraction of the 140 c.c. increase shown by the interstitial fluid. Clearly one must conclude that in addition to the obvious œdema of the cæcum and adjacent parts of the intestine, there is widespread latent or occult œdema.

One of the most interesting findings is the localisation of the œdema very largely to the cæcum and neighbouring intestine. The 50 per cent. increase in weight of the cæcum is due entirely to œdema. Localised œdema is by no means uncommon in pathological processes. Cameron, Courtice and Short (1947-48) found pulmonary œdema in Lewisite poisoning, not only when Lewisite was given by inhalation but also on intravenous injection. Œdema develops around the duodenum and pancreas when whooping-cough toxin is injected intravenously (Evans and Maitland, 1937). Many other examples might be cited. The œdema in dysenteric toxæmia cannot be due to a structural peculiarity of the rabbit's cæcum or of its capillaries, for in such an event every substance causing increased capillary permeability should produce œdema of this region. Dysentery toxin appears to have a selective action on the cæcum of the rabbit. Penner and Bernheim (1942) demonstrated that shiga toxin has no direct action on the mucosa when brought into contact with it. The intestinal lesions appear to result from vascular damage, a conclusion reached by Beitzke (1917), Hart (1918), Lorentzen (1922-23), Pick (1926) and Letterer (1944). Doerr (1907) and Felsen (1936) suggested that dysentery toxin is excreted and perhaps absorbed and re-excreted in the cæcum.

A regular chain of events thus appears to follow the injection of shiga toxin into rabbits. The capillaries of the large gut, which are selectively injured by shiga toxin, become permeable to proteins, which therefore diffuse out with the fluid. Loss of plasma proteins results in a lowering of the colloid osmotic tension and consequent diminution in the ability to retain water within the circulating blood. As a result fluid passes from the blood into the tissue spaces, where it collects as œdema, most often occult.

Felsen presented evidence to show that "extra-enteric manifestations" in human dysentery may outweigh the intestinal lesions and dominate the clinical picture. A shock-like state is not infrequently encountered in this disease. It appears likely that the findings in my experimental animals may have their counterpart in the pathology of human bacillary dysentery.

#### SUMMARY

Intravenous injection of dysentery (shiga) toxin in the rabbit leads to a reduction in plasma volume and a fall in plasma proteins, suggesting an increase in capillary permeability. At the same time



there is an increase in interstitial fluid, due partly to œdema of the cæcum, where the shiga toxin appears to localise. These effects of vascular damage can be prevented by early administration of antitoxin.

I wish to thank Prof. G. R. Cameron, F.R.S., who suggested this investigation, for his continued interest and help, Dr F. C. Courtice, who taught me the technique of blood-volume estimation, and Drs K. K. Cheng and J. D. Judah and Mr F. J. Crew for help at various times during the work. Expenses were met by a grant from the Graham Research Fund, University of London.

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# ACUTE PARIETAL ENDOCARDITIS IN A CASE OF STATUS ASTHMATICUS: A POSSIBLE EARLY STAGE OF LÖFFLER'S ENDOCARDITIS PARI- ETALIS FIBROPLASTICA WITH EOSINOPHILIA

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(PLATES CIII-CVI)

LÖFFLER of Zürich is well known in this country for his description (1932, 1936b) of transient lung infiltrations with eosinophilia. He has, however, also described (1936a) a syndrome of parietal endocarditis with eosinophilia which, beyond a brief mention by Harkavy (1941), has not hitherto been described in any but the Swiss and German literature. The condition appears to be rare, but the fact that five of the six cases so far reported came from northern Switzerland suggests that cases may go unrecognised elsewhere.

The case here presented is believed to be the earliest example of the condition so far recorded, and its occurrence in the presence of status asthmaticus adds further evidence for the allergic origin of Löffler's endocarditis.

## REPORTED CASES

Because of the unfamiliarity of the condition the features of the cases so far reported have been summarised in the table (pp. 622 and 623). It will be seen that the first five cases form a homogeneous group and that the sixth (Büchler's case, 1941-42) appears to represent an earlier stage of the same process.

The characteristic clinical features of the disease are congestive heart failure and eosinophilia. The *heart failure* is progressive, and is usually associated with ascites, oedema and enlarged liver. The heart is often not enlarged and the findings on physical and electrocardiographic examination are quite insufficient to account for the failure. Löffler has pointed out the resemblance in this respect of the clinical picture to that of Pick's disease. All the cases seen have shown what is generally called right-heart failure, though in some the left ventricle was more affected than the right.

The *eosinophilia* is sometimes very high (35,900 per c.mm. in Büchler's case) and is nearly constant, but it was absent in the earliest stage of Mumme's case (1940) and slight in the latest stage of Löffler's case I. Löffler believed that the disappearance of eosinophilia in his case indicated cessation of activity, and that his patient died of the purely mechanical sequelæ of a healed disease.

The post-mortem findings are those of massive endocardial fibrosis and extensive mural thrombosis of one or both ventricles: the valves are little if at all involved. The scar tissue and thrombus together obstruct ventricular

TABLE.—*Reported cases of parietal*

Author	Sex and age	Total duration	Presenting symptoms	Main clinical features	Blood findings
Löffler (1936a) Case I	F. 45	1 year	Anorexia; precordial pain; loss of weight	Progressive congestive heart failure without enlargement of heart; edema, ascites, etc.; lung infarcts and skin hemorrhages; afebrile	Eosinophils 5400/c.mm. at first, rose to 12,600 and finally fell to 390; E.S.R. 21 mm./hr.; blood cultures negative
Löffler (1936a) Case II Deus (1916)*	M. 37	21 months	Weakness and loss of weight	Steadily advancing congestive heart failure, with heart normal to physical examination	Constant eosinophilia, maximum 8330/c.mm.
Mumino (1940)	M. 25	2½ years	Transient vasomotor and "neurological" attacks (disseminated sclerosis suspected)	Irregularly progressive congestive heart failure; heart enlarged; rasping systolic and diastolic murmurs; B.P. 160/110; crops of purpuric spots; afebrile	Blood normal at first, eosinophilia appeared five months after onset and remained constant: maximum 18,300/c.mm.; E.S.R. normal; blood cultures sterile
Roulet (1944)	M. 48	10 months	Dyspnea on exertion	Progressive congestive heart failure without enlargement of heart	Eosinophilia constant, reaching 12,200/c.mm.
Egger (1944)	F. 49	? 1 month	Dyspnea on exertion	Rapidly progressive congestive heart failure; terminal femoral thrombosis	Eosinophils 1170/c.mm.
Büchler (1941-42)	M. 44	? 2 weeks	Urticaria; terminal heart failure	Congestive heart failure developing rapidly in last week or two of life	Constant high eosinophilia reaching 35,900/c.mm.

\* Deus had published this case in 1916, but, because *Strep. viridans* was cultured from

*endocarditis with eosinophilia (Löffler)*

Other significant diseases present	Post-mortem findings			
	Right ventricle	Left ventricle	Rest of heart	Other organs
...	Massive fibrosis of whole endocardium, with organising thrombus on the surface	Massive fibrosis of endocardium of antero-inferior half, with organising thrombi on surface	Scarring of endocardium of right auricle; moderate fibrosis of mitral cusps	...
...	Massive fibrosis of endocardium, with obliteration of most of cavity	Partially organised mural thrombi filling distal half of cavity	...	...
...	Massive fibrosis of endocardium of distal half, with organising thrombus on surface	Similar to right	Thickening of mitral and tricuspid cusps, which bore warty vegetations	Organising thrombus on intima of whole aorta; "endarteritis" of small arteries in most viscera including lungs and brain (see Discussion, p. 627)
...	Organising thrombus of whole endocardium, nearly filling lumen; histological evidence of activity, with infiltrations including eosinophils	Normal	Under surface of tricuspid cusps involved by endocardial lesion	"Eosinophil granulomata" in walls of splenic arterioles and veins, and what is called a focal embolic nephritis of Löhlein
Rheumatoid arthritis for ten years and recurrent tonsillar abscesses	Thin layer of organising thrombus on half of endocardium	Laminated thrombus 2.5 cm. thick nearly filling cavity, and based on a 5 mm.-thick fibrous endocardium	Many myocardial scars	Thrombosis of both femoral veins; active tuberculosis of mediastinal lymph nodes
Repeated attacks of "urticaria papulosa necroticans" for six years, with achlorhydria, anaemia and episodes of perihepatitis and pleurisy; enlarged spleen removed without effect	Normal	Recent thrombus without organisation over whole endocardium; dense subendocardial infiltration of polymorphs, eosinophils, lymphocytes and plasma cells	Many myocardial scars and foci of cellular infiltration, including eosinophils	"Eosinophil infiltration" of lungs and liver; active tuberculosis of peribronchial lymph nodes; early cirrhosis of liver; scar of a duodenal ulcer

contraction from within in much the same way as the thickened pericardium of Pick's disease hinders it from without, and adequately explain the type of heart failure present. Histologically, the findings have been in general simply those of old scar tissue and organising thrombus: in only two cases (Egger's and Büchler's) were there significant signs of activity in the way of cellular infiltrations in the endocardium. Signs of rheumatism have been uniformly absent.

### REPORT OF THE PRESENT CASE

V. C., a spinster, was first admitted to Hammersmith Hospital in 1942 at the age of 47, with anemia. She gave a history of chorea at the age of 20, a suspected duodenal ulcer at 31, hæmoptysis at 33 and achlorhydric hypochromic anemia at 44. She had no history or family history of asthma, urticaria, hay fever, rheumatism or heart disease. The recurrence of anemia (Hb. 6.2 g., C.I. 0.53, leucopenia, no eosinophilia) which brought her to hospital was treated successfully with iron. While in hospital she had her first asthmatic attack, and was readmitted twice in the autumn of the same year in severe attacks precipitated by respiratory infection. Investigation of the cardiovascular and respiratory systems demonstrated no abnormality between attacks. No exogenous allergen was demonstrated, but *Streptococcus viridans* was found (as on many subsequent occasions) in the sputum. Eosinophilia was absent. Psychotherapy proved helpful and for the next three-and-a-half years, though having frequent attacks, she was not incapacitated.

In October 1946 she was in hospital for three weeks with status asthmaticus following a cold. In June 1947 she was readmitted in the same condition and this time did not respond to treatment. On admission breathing was typically asthmatic and there were loud rhonchi all over the chest. The sputum was abundant and frothy and contained polymorphs with an occasional eosinophil. The heart was of normal size and its sounds, as far as they could be heard, were normal. The jugular venous pressure varied greatly with respiration, reaching a maximum of 6 cm. above the sternal angle. Cardiac catheterisation showed pressures (of water above the sternal angle) of -14 cm. in the right atrium and +7 cm. in the right ventricle. These are within normal limits: it was concluded, therefore, that the raised jugular pressure was the effect of abnormal intrathoracic pressures and not a sign of cardiac failure. The arterial blood was 80 per cent. saturated with oxygen. The E.C.G. on admission (fig. 1) showed only the spiked P waves of cor pulmonale, which were constant in and out of attacks: but in a series taken in the last week (fig. 2) a flattening of T in  $V_6$ , sparing  $V_3$  and  $V_1$ , was thought to indicate some lesion of the left ventricular myocardium.

The blood during this last attack showed a moderate but definite and sustained eosinophilia, ranging between 500 and 700 per c.mm. on four counts. This is the more significant in view of the fact that numerous counts during previous attacks had never shown more than 87 eosinophils per c.mm.

Treatment proved practically without effect. The drugs given during the last attack included adrenaline, ephedrine, stramonium, theophylline and pethidine, but no sulphonamides or serum of any kind. She died on 27.6.47, four weeks after admission.

### Post-mortem examination

This was carried out 19 hours after death. Apart from two large cavernous hæmangiomas in the liver, findings of interest were confined to the lungs and heart.

## ACUTE PARIETAL ENDOCARDITIS

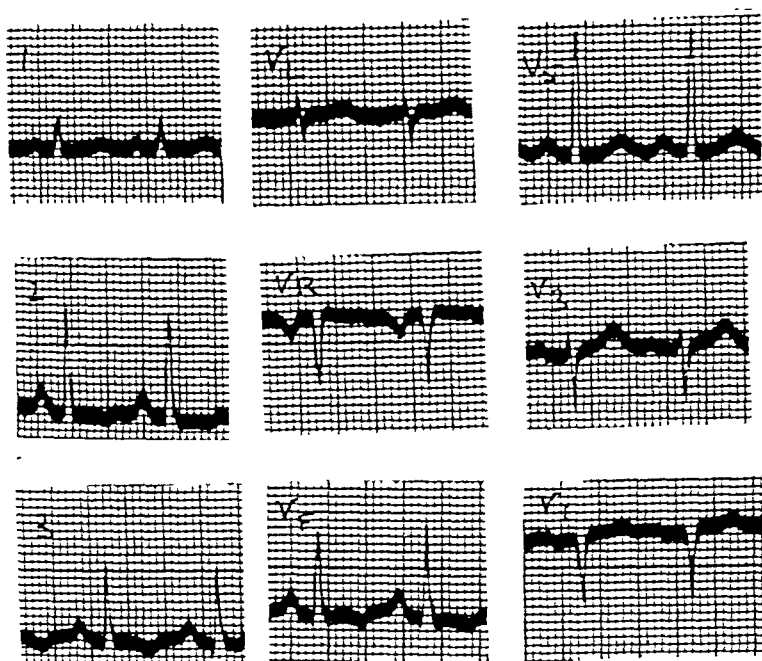


FIG. 1.—E.C.G. on 3rd July 1948. Cor pulmonale (spiked P waves in leads II and III) and vertical heart (unipolar leads) are the only significant changes. Note a well-developed positive T wave in  $V_5$ .

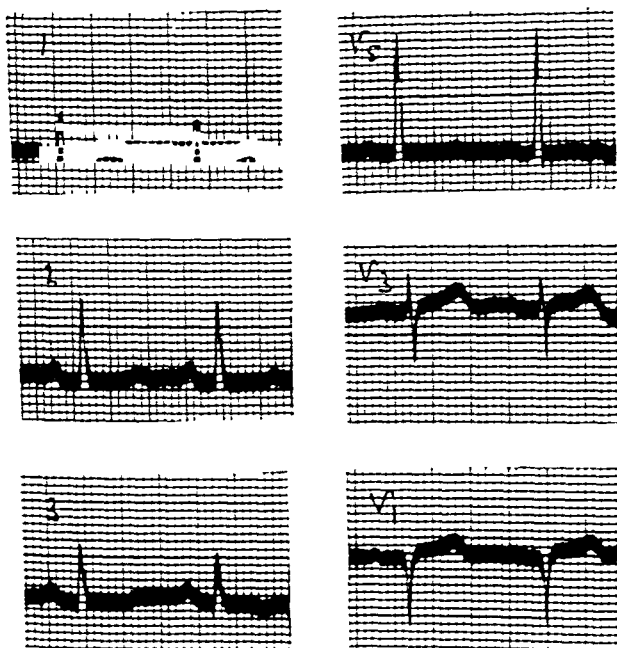


FIG. 2.—E.C.G. on 26th July 1948, three days before death. No general change, but the T waves are now very much flattened in  $V_5$  though not affected in  $V_3$  and  $V_1$ : this was believed to indicate left ventricular myocardial damage.



## ACUTE PARIETAL ENDOCARDITIS

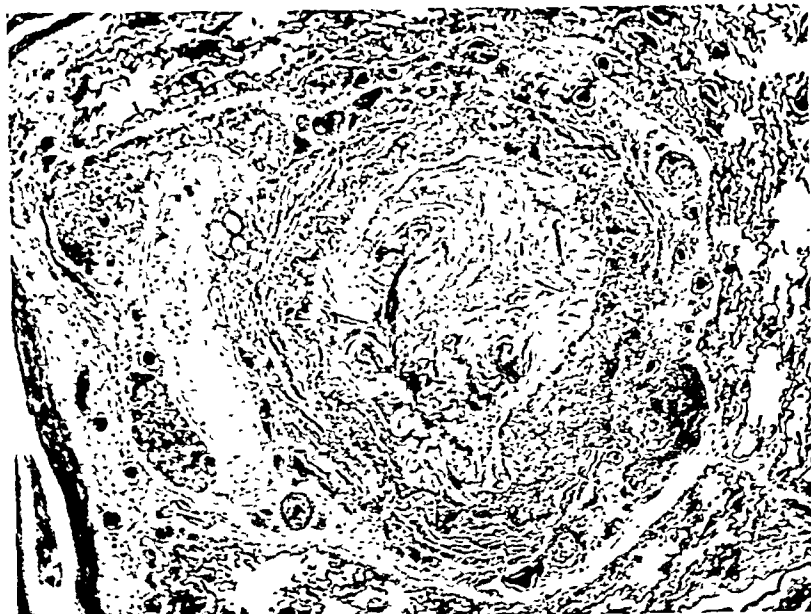


FIG. 3.—A small bronchus plugged with mucus. On the left, between the cartilage and the pulmonary artery, there are hyperplastic mucous glands. Hæmalum and eosin.  $\times 25$ .

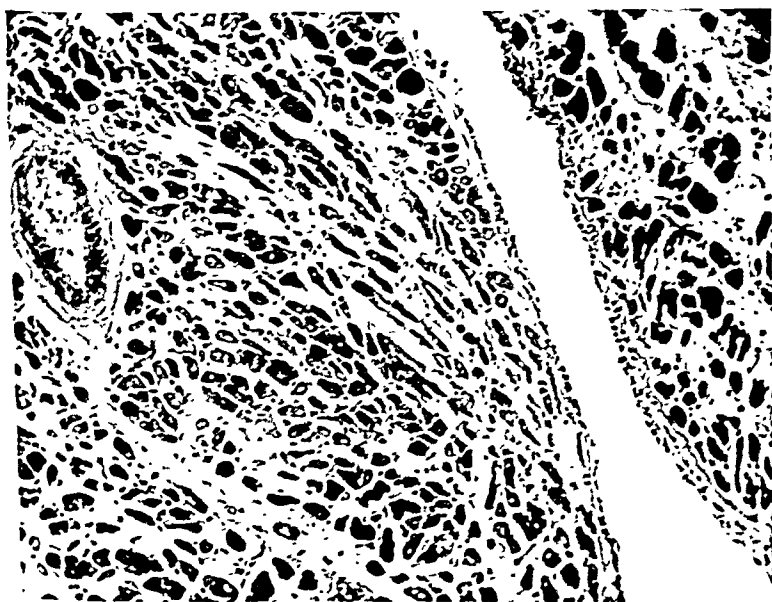


FIG. 4.—A crypt between columns carner of the left ventricle, showing myocardial oedema, a normal coronary arteriole and the endocardial lesion. The left hand wall of the crypt shows at its upper end the earliest stage of the process. Hæmalum and eosin.  $\times 110$ .





## ACUTE PARIETAL ENDOCARDITIS

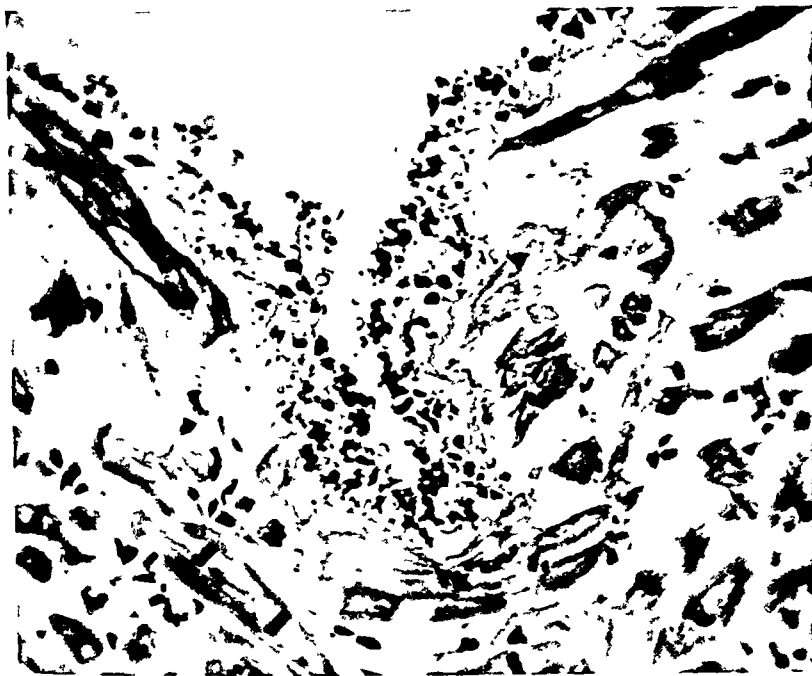


FIG. 5.—Endocardium of left ventricle, showing a more advanced stage of the lesion. At this point some spread of inflammatory cells into the subjacent myocardium has occurred. Hæmalum and eosin.  $\times 270$ .



FIG. 6.—Endocardium of left ventricle, showing swollen endothelial cells, many mononuclear cells with pyknotic nuclei and some polymorphs and lymphocytes. Hæmalum and eosin.  $\times 580$ .



ACUTE PARIETAL ENDOCARDITIS

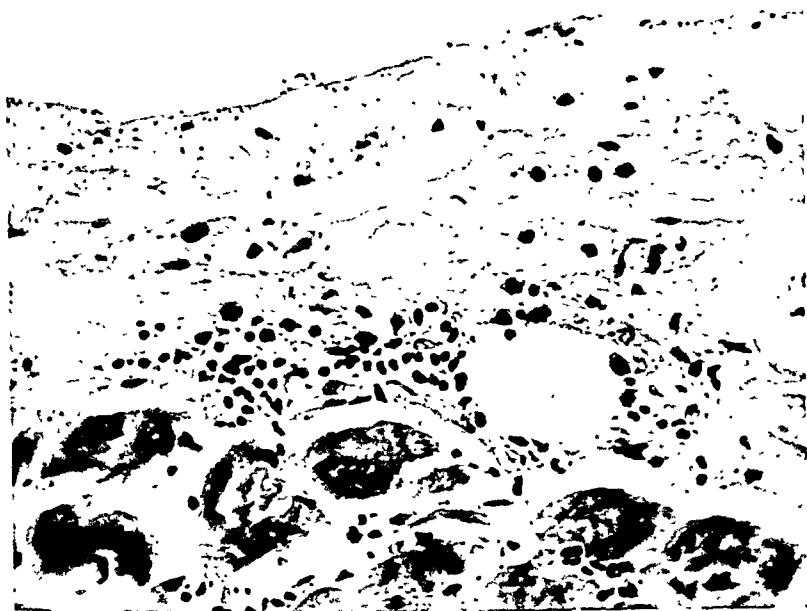


FIG. 7.—Pericardium over left ventricle, showing loose infiltration of cells similar to those in the endocardium, but with more lymphocytes, near a small vessel. This is the largest lesion seen. Hæmalum and eosin.  $\times 270$ .

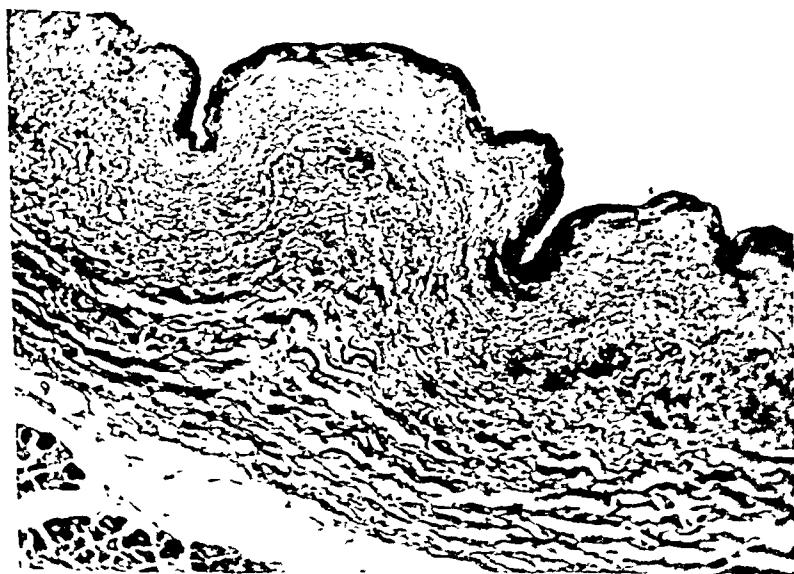


FIG. 8.—Endocardium of posterior wall of left atrium, showing a thin layer of surface fibrin (dark) without cellular reaction. Picro-Mallory.  $\times 130$ .



The lungs were of average size and not macroscopically emphysematous, though microscopically there was considerable evidence of breakdown of alveolar walls.

The heart weighed 340 g. and was externally normal except for excessive sub-epicardial fat. There were a few asphyxial petechiae on the parietal pericardium. The right side of the heart was moderately dilated. The right ventricular wall was 4 mm. thick and infiltrated with adipose tissue to an exceptional degree. The left ventricle was not dilated, its wall was 1.2 cm. thick, the myocardium soft and pale, the endocardium macroscopically normal. The mitral valve showed some pin-points of calcification in the contact line and minimal thickening of a few chordæ: the other valves and the coronary arteries were normal.

### Histology

*Lungs.* At least three-quarters of the smaller bronchi (fig. 3) are obstructed by thick mucous plugs which manifestly had been the main cause of her respiratory distress and her death from asphyxia. Other changes often seen in fatal asthma (Thomson, 1945) such as eosinophilic infiltration of the submucosa and of the mucous plug and thickened hyaline basement membranes are present, but there are no Curschmann's spirals or Charcot-Leyden crystals. The mucous glands of the bronchioles are markedly hyperplastic. The bronchial muscle is normal in amount. There is no pneumonia and no vascular or granulomatous lesions.

*Heart.* The wall of the left ventricle shows lesions more striking than was to be expected from the naked-eye findings. The endocardium is chiefly involved: the change is widespread but best seen in the depths of the crypts between the columnæ carneæ, especially in the region of the anterior papillary muscle. The earliest recognisable lesion is swelling and irregularity of the endothelial cells of the endocardium (fig. 4). Their nuclei seem to have become rapidly pyknotic and have begun to break up. In more advanced lesions (figs. 5 and 6) large numbers of similar cells have collected in the line of and immediately beneath the endocardium. As to whether these are endothelial cells proliferated locally or histiocytes which have wandered in, no certain answer is possible, but no mitoses are seen. In all but the earliest lesions polymorphs are present in moderate numbers. There are also a few lymphocytes and very occasionally an eosinophil or plasma cell. A few rather prominent cells of uncertain nature are also present, with a single round eccentric nucleus and deeply eosinophilic, almost hyaline cytoplasm: they are not necrotic muscle fibres, as they can also be seen in the pericardium. No Anitschkow myocytes are seen. There is a little fibrin on the surface of some lesions, but no extensive thrombosis. The myocardium is cedematous and its fibres, though not fatty, stain very irregularly, but apart from an occasional minimal invasion by the endocardial infiltration it

## SUMMARY

1. In a woman of 53 dying of status asthmaticus, the endocardium of the left ventricle showed extensive cellular infiltration. There was an accompanying blood eosinophilia of 500-700 cells per c.mm.

2. This is believed to represent the earliest stage of the endocarditis parietalis fibroplastica with eosinophilia of Löffler.

3. The six previously published cases of this syndrome are summarised.

Dr J. G. Scadding was in charge of the case, and he and Dr J. Crofton first acquainted me with Löffler's work. Dr Paul Wood interpreted the electrocardiographs, and Dr E. P. Sharpey-Schafer the cardiac catheter studies. Professor J. H. Dible, Dr C. V. Harrison and Dr I. Doniach have assisted in the preparation of this paper. Mr J. G. Griffin was responsible for the sections and Mr E. V. Willmott for the photomicrographs. To all these, and to Professor Löffler for his opinion on the case, my thanks are due.

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## SHORT ARTICLES

616.71—018.46:616.469—002.198

### THE BONE MARROW IN GLANDULAR FEVER

A. COLIN P. CAMPBELL

*From a Royal Air Force General Hospital and the Department of  
Pathology, Edinburgh University*

(PLATE CVII)

Descriptions of the remarkable blood picture in glandular fever abound *ad nauseam* and of recent years some knowledge of the tissue changes has begun to accumulate, but there is a peculiar absence of agreement on the question of whether, and how, the bone marrow is involved. Using sternal puncture, Morrison and Samwick (1939), Israël (1941), Halcrow *et al.* (1943) and Stiefel (1943, quoted by Bethell *et al.*, 1944) report an increase of lymphocytes in the marrow. Other workers, *e.g.* Vogel and Bassen (1939), Mendell *et al.* (1942) and Limarzi *et al.* (1945), have found merely a myeloid hyperplasia with inhibition of granulocytic maturation and no sign of a lymphoid reaction. Most of these studies have been made on smears, and in these circumstances reports of lymphoid cells in the marrow must be looked on with suspicion, since it appears to be widespread practice to make "marrow" smears, not of the solid marrow particles, but of the fluid aspirated, which consists largely of blood from the vessels of the marrow. This blood contamination could introduce into the smears many of the characteristic "glandular fever cells." A satisfactory answer to the question of marrow involvement can only be given by histological examination.

Even histological examination, however, has resulted in conflicting reports. Freeman (1936) found by sternal biopsy, in a single case, a remarkable degree of infiltration of the marrow by mononuclears, many of them resembling the characteristic blood cells of this disease. Du Bois (1930), reporting what appears to have been the first autopsy in a case of glandular fever, noted the presence in the marrow of large mononuclear cells; he gave, however, little or no detail. Recently Custer and Smith (1948) have reported no less than nine cases with autopsy; they confirm the presence in some cases of myeloid hyperplasia, but have found no abnormal cells in the marrow apart from those of the circulating blood within the vessels.

In view of this discordance in the literature it seemed worth while to report my findings in a series of cases in which the marrow was examined by both smears and sections.

#### *Material and methods*

Sternal puncture was carried out in a series of typical cases of sporadic glandular fever occurring in young adults, members, chiefly male, of the Royal Air Force. Smears and paraffin sections were made from the marrow flecks (Campbell, 1948). Adequate sections were obtained in 15 cases. They were stained either by Turnbull's Jenner method (Turnbull, 1931) or by Barrett's method (Barrett, 1944).



To assist in the correlation of marrow cytology with that of the peripheral blood (the comparison of the abnormal lymphocytes as seen in smears of the blood with presumably identical cells in sections of the marrow being by no means easy), in each case paraffin sections were prepared of white blood-cell concentrations. About 1 c.c. of venous blood was withdrawn, heparinised with a trace of heparin powder and centrifuged in a narrow tube closed at the bottom with a rubber cork. The supernatant plasma was pipetted off and fixative similar to that used for the marrow gently pipetted on to the surface of the leucocyte layer. After about 6 hours the cork was removed from the bottom of the tube, and the red-cell layer washed away with water. The leucocyte layer could then be pushed out as a solid disc and embedded and cut in paraffin.

Only one marrow examination was made in each case, usually about the peak but sometimes in the early stages of the decline of the lymphoid reaction in the blood.

### *Results*

In 8 out of the 15 cases the marrow showed obvious hyperplasia; in 2 cases cellular marrow had ousted almost all the fat. The hyperplasia was leucoblastic, normoblasts being relatively reduced. There was no obvious correlation between the degree of hyperplasia and the degree of agranulocytosis or of "shift to the left" in the peripheral blood.

Apart from this hyperplasia, which has been noted by most workers who have examined the marrow, more specific lesions were seen in 12 of the 15 cases. These consisted of focal proliferations of mononuclear cells (figs. 1 and 2). They varied in size, the smallest recognisable foci being about 80  $\mu$  in diameter and the largest about 500  $\mu$ . They were numerous in 4 cases only; in 8 others they were sparse and had to be looked for with care. The amount of marrow available in such sections is of course small; possibly larger sections would have shown the characteristic foci in all the cases.

These foci were composed chiefly of two cell types. (1) Fairly large cells, with large, oval or irregularly elongated, pale vesicular nuclei and one or two prominent nucleoli; their cytoplasm was abundant and faintly basophil; their outline was ill-defined and irregular; sometimes they appeared to be syncytial, but their outline was too faint for this to be definitely established. These cells had the appearance of undifferentiated reticulum cells. In some of the foci where they were numerous they suggested the endothelioid cells of the tubercle follicle, though their grouping was never as compact or as homogeneous. Occasionally they showed mitoses. No phagocytosed material could be seen in them. (2) Smaller cells, of the same size and much the same appearance as the medium-sized lymphocytes of the germinal centres in normal lymph nodes. These cells had a more definite, usually rounded outline, and a smaller nucleus, with a denser chromatin network, round or more commonly irregularly indented; nucleoli could not be identified with certainty. Some of these cells appeared identical with the medium-sized lymphocytes of normal lymphoid germinal centres; others showing more cytoplasm and a slightly darker-staining nucleus appeared identical with the "glandular fever cells" as seen in the sections prepared from the peripheral blood. Transitional forms were also encountered.

In addition to these two main types, occasional large cells resembling the large lymphocytes of normal lymphoid follicles were seen, and some of the foci also contained small numbers of plasma cells, usually in groups of two or three.

The constitution of the foci varied from case to case. In cases examined at the peak of the lymphoid reaction in the peripheral blood, reticulum-cell proliferation predominated; in cases examined during decline, the second

## BONE MARROW IN GLANDULAR FEVER

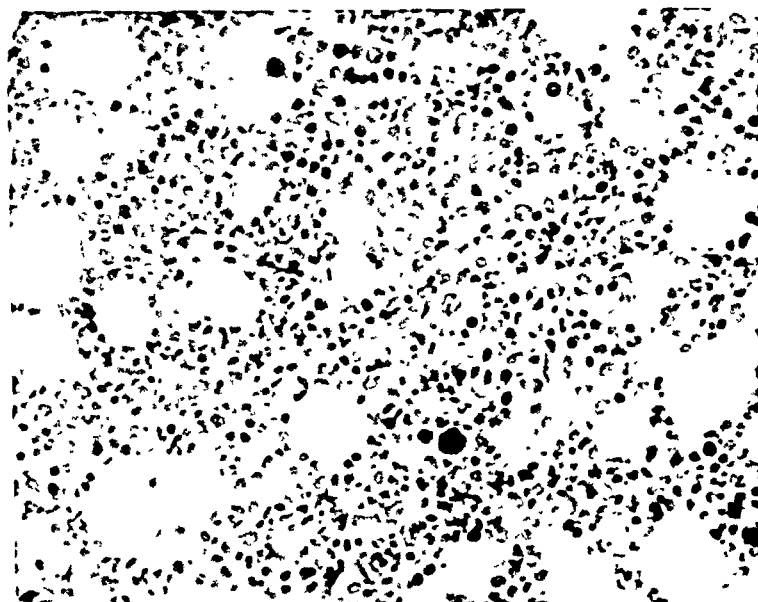


FIG. 1.—Glandular fever. Section of sternal marrow, showing a characteristic focus of lymphoid hyperplasia top right of centre. Barrett's stain.  $\times 250$ .

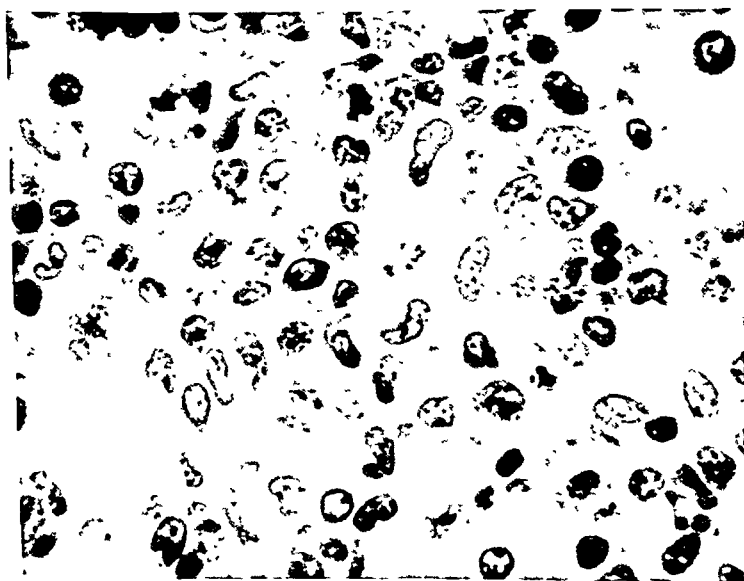


FIG. 2.—High-power view of the same focus, showing the characteristic admixture of reticulum cells, some of which are of endotheloid type, and lymphocytes of various stages of maturity. Barrett's stain.  $\times 800$ .



cell type—lymphoid cells, including both immature forms and the more mature abnormal lymphocytes as seen in the blood—tended to be more prominent.

These foci never showed the sharply defined outline characteristic of normal lymphoid germinal centres. They were also quite distinct from the collections of small lymphocytes which one occasionally sees in the marrow in a variety of pathological states, and since such lymphocytic foci are numerically sparser in the average bone marrow than are the characteristic foci in the typical glandular-fever marrow, it appears probable that the latter are produced *de novo* by lymphoid metaplasia of the marrow reticulum cells rather than by activation of pre-existing lymphoid foci.

### Discussion

These foci of lymphoid reaction in the marrow are obviously similar to the hyperplastic lymphoid tissue which occurs throughout the body in glandular fever. The pathology of the lymph nodes in this disease has been frequently described, notably by Downey and Stasney (1935-36) and Gall and Stout (1940). In both of these papers, the hyperplasia both of undifferentiated reticulum or "stem" cells and of lymphoid cells, mature and immature, is emphasised. Gall and Stout describe proliferation of clasmatoocytes as well as of stem cells, sometimes forming foci resembling groups of endothelioid cells—an appearance I have noted in the marrow foci. Downey and Stasney regard the formation of lymphocytes in glandular fever as essentially similar to that in normal lymphoid tissue—from reticulum cells through progressively maturing stages of lymphocyte, the intensity of the stimulus producing however an unusual degree of reticulum-cell proliferation. They contrast this with the lymphoid hyperplasia of acute lymphatic leukaemia, where the striking feature is proliferation of lymphoblasts. Lymphoblasts of the leukæmic type are not a feature of the glandular fever reaction. Moeschlin (1940) and Tischendorf (1944) came to essentially similar conclusions.

Lymphoid hyperplasia, infiltration or metaplasia has been described in various other organs in glandular fever. It has been noted in spleens surgically removed because of spontaneous rupture (King, 1941; Smith and Custer, 1946; Timmes *et al.*, 1948; and others). It has also been described, in fatal cases, in liver, spleen, lymph nodes and bone marrow (Du Bois); in liver, kidneys and spleen (Ziegler, 1944); and in liver, kidneys, heart, lungs, adrenals, pancreas, stomach, intestine, prostate, testes, hypophysis, meninges, brain, spinal cord and peripheral nerves (Custer and Smith).

I have not seen a similar picture in the marrow in any other condition. The marrow infiltrations in lymphatic leukaemia show a more uniform lymphoblastic or lymphocytic infiltration without the striking degree of admixture with reticulum cells and cells of endothelioid appearance. Whether such foci occur in other diseases characterised by a lymphoid reaction, such as rubella and infective hepatitis, I cannot say; I have not seen them in the very few marrows from these diseases which I have examined.

These foci are difficult if not impossible to demonstrate by the smear technique. I could not identify the participating cells in any of the smears in my cases, probably for two reasons; first because the foci are usually sparsely distributed in the marrow and may not be present in the particles chosen for smearing, and second because the cells making up the foci, including as they do many fixed reticulum cells, will tend to be disrupted and destroyed in the act of making the smears. Certainly there is no likelihood of demonstrating the typical changes in the marrow by smears of the fluid blood aspirated. These lesions emphasise the desirability of examining sternal puncture bone marrow in paraffin sections as well as in smears.

In view of my findings it is difficult to explain the report of Custer and Smith that the bone marrow in nine fatal cases showed no lymphoid reaction.

Possibly their material, collected from various sources and perhaps under difficult military circumstances, may have suffered from post-mortem change and less than optimal fixation. In most of my cases the characteristic foci were small and sparse and might well have escaped notice under such conditions.

### Summary

Bone marrow obtained by sternal puncture was examined histologically in fifteen cases of glandular fever. Twelve cases showed foci of atypical lymphoid hyperplasia such as have not been seen in the marrow in any other condition.

I am indebted to the Director-General of the Medical Branch of the Royal Air Force for permission to publish this report and to Group-Captain C. A. Lindup, commanding officer of the hospital, and Wing-Commander E. H. Hudson, medical specialist, for access to the cases.

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THE HISTOLOGICAL PREPARATION OF BONE-MARROW PARTICLES,  
UTERINE CURETTINGS AND OTHER SMALL TISSUE FRAGMENTS

A. COLIN P. CAMPBELL

*From the Pathology Department, Edinburgh University*

Many histologists have evolved methods for taking small tissue fragments safely through the steps of paraffin embedding. The method described below, however, seems to offer certain advantages: it is reasonably free from "finickyness," it inflicts a minimum of forceps trauma on the tissue and it facilitates orientation of the small fragments in the block. No doubt others have evolved and used a similar method, but if so I have not seen it described.

The method is especially useful for the small bone-marrow particles obtained by sternal puncture and I have used it also as routine for uterine curettings. It consists essentially of embedding the particles in agar after fixation, thus obtaining a single block which can be easily taken through all the subsequent steps.

*Procedure for sternal marrow fragments*

It is my custom to aspirate 2 or 3 c.c. of blood, which is discharged into a small test-tube containing a tiny knife point (about 1 sq. mm.) of heparin powder and gently mixed. It is then transferred with a Pasteur pipette to a large Petri dish and out-spread as widely as possible. The tiny marrow flecks can be easily seen if the blood is gently run to and fro by tilting the dish. Some flecks are first picked out with a Pasteur pipette for making smears; a selection of the rest is then picked out and transferred to a small glass dish or bottle. The blood transferred with the flecks is as far as possible removed with the pipette and fixative is pipetted into the receptacle. I have found that a mixture of 4 parts of corrosive-formol (10 per cent. formalin in saturated aqueous mercuric chloride) and 1 part of methyl alcohol gives good fixation in 4-6 hours. The fixative is then pipetted off and water added. After washing by gentle agitation for a few seconds the greater part of the water is pipetted off and the residual drop or two, carrying the tissue fragments, is transferred to a glass slide. The excess water is removed with the edge of a filter paper, the marrow fragments are pushed together into a compact group, and a glass ring of suitable size, with a flat-ground lower rim, is placed around them. A tube of 2 or 2½ per cent. agar is melted down in a water-bath or by gently heating over a Bunsen flame and cooled under the tap to about 50° C. With a Pasteur pipette enough melted agar is run into the glass ring mould to form a layer 5 or 6 mm. deep above and surrounding the tissue fragments. It is helpful to warm slightly the slide bearing the mould and the tissue before adding the agar, so that the latter will not set too quickly, before it has had time to penetrate between the fragments. When adding the agar, it should be gently run in by holding the pipette point to the side of the glass ring so as to disturb the fragments as little as possible. If, as occasionally happens, they rise with the surface meniscus, they can be pushed down to the slide with a needle before the agar sets. The slide is left on the bench for a few minutes to allow setting. It is then wetted, the glass ring is slid off it, and the agar block is pushed out and trimmed. This block, holding the marrow fragments, can be handled with forceps (avoiding coarse pressure) and taken easily through the succeeding stages of washing (unnecessary with the fixative mentioned above), dehydration, clearing, paraffin saturation and embedding. The agar interferes with none of these procedures. Sections are cut in the ordinary

way; they tend to flatten out on warm water less easily than sections from blocks without agar and may need judicious poking with a needle. They stick to the slide quite satisfactorily.

*Procedure for uterine curettings and similar fragments*

The procedure here is essentially the same, but, as one may be dealing with a considerable number of larger fragments, I have found it more convenient to use as a mould two angled slips of glass, instead of a glass ring. These can easily be made by cutting from a  $3 \times 1$  in. glass slide two slips each about  $1\frac{1}{2}$  in. long and  $\frac{1}{2}$  in. wide, bending them by heat to a right angle  $\frac{3}{4}$  in. from one end and grinding one edge and the two ends flat on a carborundum stone. These can be used to make a mould of varying size, similar to the brass embedding moulds of Leuckhart, so long in use for paraffin embedding.

For curettings and such fragments, apart from the advantage of reducing the material to a single easily handled block, the method has the following additional virtues. (a) By blocking a series of curettings from different cases in blocks of different sizes and shapes or marking the blocks by cutting off one or more corners, blocks representing several different curettings can be taken through the various stages together without the need for using individual reagent bottles. (b) It is much easier to orientate the fragments neatly and correctly when one is manipulating them at leisure on a dry slide, than it is when embedding them in hot paraffin which is constantly tending to cool and cloud.

I have used the method also for the convenient handling of numbers of blocks of different organs from small animals such as mice. Ten or twelve different organs can be taken through in perhaps two blocks. The various pieces of tissue should be set as close together as possible, as the more agar there is left between the pieces, the greater is the difficulty in inducing the sections to flatten out on warm water. I have not found that the agar interferes in any way with staining; it may stain very lightly with hæmatoxylin or other stains, but never enough to prove a nuisance. This faint background staining only occurs around and not through the tissue, as the agar surrounds and holds together but does not impregnate the fragments.

A histologist who has access to a bacteriological store cupboard need not bother to make up his own agar solutions; standard bacteriological nutrient agar works quite satisfactorily; the broth contained therein seems to have no adverse effect on the tissue fragments. The glass moulds can be home-made with a minimum of skill in glass working. The angle pieces have already been described. The rings may be made of several diameters; I have found it desirable to have a series ranging from about 6 mm. to 2 cm. in internal diameter, the smaller sizes being used for sternal puncture material.

## OBITUARY NOTICES OF DECEASED MEMBERS

### Edward Ffolliott Creed

Born 6th May 1893. Died 27th September 1947

(PLATE CVIII)

EDWARD FFOLLIOTT CREED, the second son of the late Rev. C. J. Creed, died suddenly after a myocardial infarction on 27th September 1947, at the early age of 54. He had survived one previous attack some months before and, at the time of his death, was recuperating on holiday.

Creed was born in Leicester, where his father was vicar of All Saints' Church. He went to the Wyggeston Grammar School whence he obtained a Millard Scholarship to Trinity College, Oxford, in 1910. Here he took honours in chemistry in the final school of natural sciences and was awarded a Burney Yeo scholarship to King's College Hospital in 1913. He obtained the B.M. degree in 1916, M.R.C.P. in 1923, D.M. in 1927, and was elected F.R.C.P. in 1932.

After qualifying, Creed entered the Army and served in Sierra Leone in the R.A.M.C. with the rank of captain. On his return to civil life in 1919, his early training in scientific work, as well as his personal tastes, led him to choose the laboratory as his career. He was appointed bacteriologist at his own hospital and, on the retirement of Dr d'Este Emery in 1921, became Director of the Department of Pathology at the remarkably early age of 28. To his colleagues, the appointment seemed so natural that he slipped into the post without serious outside competition and thereafter set a standard of sound systematic teaching in the medical school, which bore much fruit during the twenty-six years of his service. Among his pupils can be counted Terence East, Wilfred Sheldon, Harold Edwards, Charles Newman, James Livingstone, R. A. McCance, J. M. Cummings and J. V. Dacie.

There can be little doubt that Creed's name will survive in the memory of his own beloved hospital for many a long year, for his finest work was done there with his junior staff and the many generations of his own students. His honesty in work was but a reflection of his own unswerving ideals, which were so direct and straight that he could scarcely comprehend dishonesty and deceit in ordinary life or a biased interpretation of an equivocal experiment in professional work. His bent and inspiration were the precise procedure, the exact technique, the meticulous method and the faultless performance.

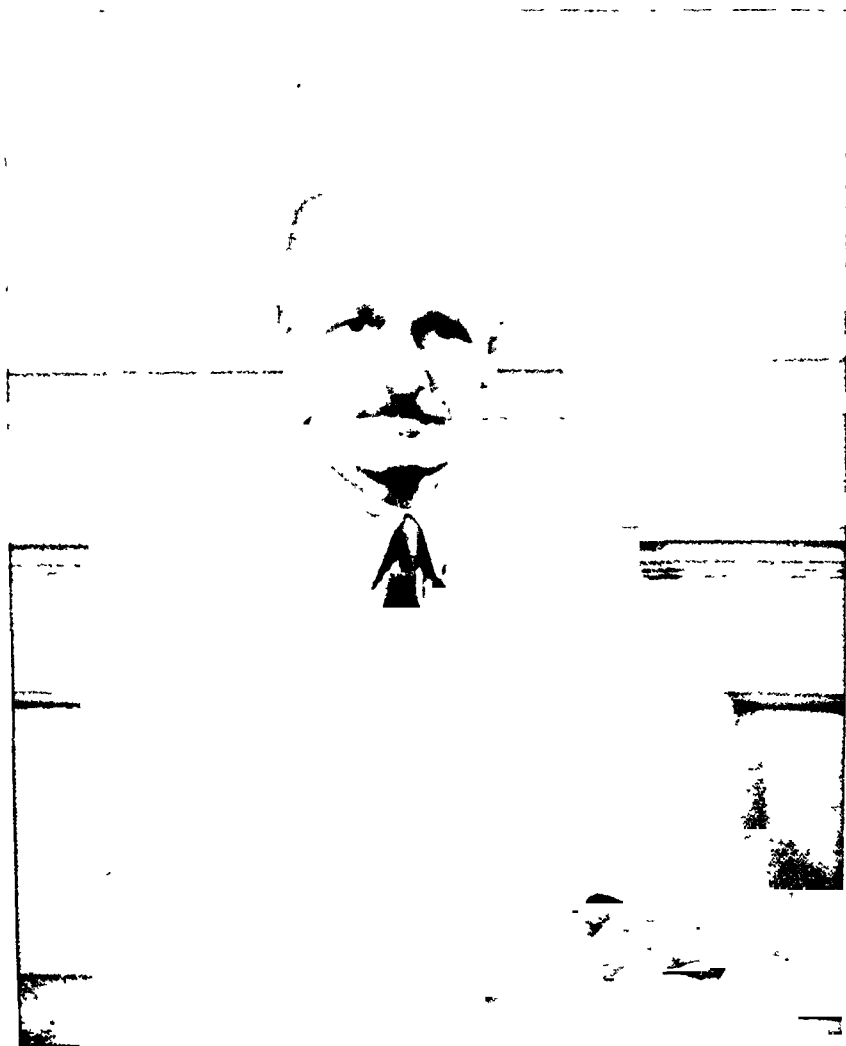


All of these he could impart to his juniors, his trainees and his students. Only those who have incorporated such discipline into their own work can appreciate how fundamental is such training at the outset of a laboratory career.

Likewise with teaching. Creed's elementary lectures were given with a lucidity which comes only from conscientious and careful preparation; they were models because of their simplicity, their emphasis upon basic principles and their logical appeal even to those poorly endowed with reasoning powers. One of his colleagues has written "For young workers in his laboratory it was a privilege to enjoy the time he would always gladly spend on demonstrating a section or explaining a technique. Nothing was too much trouble for Creed in these matters. I well remember the pains he took in revealing to me the complex mysteries of the Wassermann reaction twenty-five years ago."

Creed's main interest was in clinical pathology. He was an accomplished bacteriologist and a reputable hæmatologist. His early training in chemistry gave him a sound appreciation of biochemistry, while a long experience in post-mortem work made him a most competent histologist and morbid anatomist. This all round knowledge coupled with the clinical acumen which enabled him to take the M.R.C.P., made him the ideal clinical pathologist and of a type which is rarely seen in a large London hospital where the amount of work usually calls for separate special departments. This aspect of Creed's work, the natural linking of the laboratory to the ward, could only be appreciated in his own hospital, for such things are not written in journals or the medical press for all to read. Yet most clinical pathologists would like to have said about them what one of the physicians of his hospital wrote of Creed: "To the clinician, a chat with Creed about the pathological aspects of some puzzling case, was stimulating and instructive. His colleagues, who were all his friends, will find him hard to replace. Those informal chats in his room on a hundred and one subjects, always seasoned by his lively humour, will be sadly missed." Creed was an examiner in pathology in the Universities of Cambridge and London, as well as for the Conjoint Board. In this capacity, he was shrewd and searching, but scrupulously fair. He extracted knowledge with the same gentle and kindly air as he imparted it, so that no candidate could feel ill at ease or that he had not been given every chance. His assessment was eminently just and most helpful to his co-examiner.

Creed did not contribute greatly to medical literature, but his small output was of high quality. In matters of technique it was a revelation to go to his laboratory and to find procedures in use which might well have been published for the benefit of others, but which Creed accepted as the normal routine of a well-disciplined laboratory. His thesis for the degree of D.M. at Oxford was a most careful and critical appreciation of the many variables of the Wassermann





reaction. Had the thesis been published, it would have been universally accepted as a great contribution to the subject. As it was, very many pathologists knew of Creed's reliability in the performance of this sometimes baffling test, and he was frequently used as a court of appeal in doubtful cases. Diffidence in publication was dictated not by inertia but by an earnest wish that the article should be perfect, complete and beyond criticism—an ideal rarely attained in medical literature. There is no better example of this than his work on the fragility of the red corpuscles, which was known to the world under his name for some years before the article itself appeared in print (this *Journal*, 1938, xlv, 331-340). Creed's only other contributions to the literature were a short paper on the value of blood transfusions (*Medical Press and Circular*, 1938, cxvii, 462-465) and the articles on abortus fever and anthrax in the *British Encyclopædia of Medical Practice* (London, 1936, vol. I, pp. 68-74 and 629-642). The principles which he enunciated concerning the importance of precise technique, the necessity for controlling or standardising all the potential variables and the clinical significance of minor deviations when technique was beyond question, made a valuable contribution to the diagnosis and natural history of congenital hæmolytic icterus. Before Creed made his careful studies on the significance of saline fragility, clinicians as well as laboratory workers had always been baffled by the apparent inconsistencies of a simple diagnostic laboratory test.

On the administrative side of laboratory work, his power of clear thinking was a great asset, but he was very rigid in his ideas and found it difficult to delegate work to others. Neither did he like administration, which he found exhausting by reason of the amount of attention which his conscientious nature felt bound to devote to preparation. Nevertheless, his shrewd commonsense, clear outlook and retentive memory made him an excellent chairman of committees, so that in hospital affairs his advice was always welcome and always the product of balanced consideration combined with a competent knowledge of the facts of a case.

Terence East writes "In the hospital circle we all thought so well of him because of his absolute honesty, his soundness of judgment, and on account of the lively interest he always took in any subject upon which one approached him. Nothing could be too much trouble to him and his high standard gave good results, for he never would allow himself to be content with anything but the best. This rather meticulous ideal might perhaps have somewhat restricted his outlook and some might have thought him a little stiff and pernickety, but this was not so. One knew one could rely on getting the best from him, and the range was wide, for he had an extensive and remarkably accurate supply of knowledge, both practical and literary, on many subjects. He was not at all a dull or 'donnish' man; he had a keen and subtle sense of humour. His somewhat shy and retiring tempera-

ment, never pushing himself forward or attempting to advertise, made him less well-known than his talents deserved."

Howard Whittle writes "I was one of Creed's first trainees and early acquaintance led to life-long friendship which deepened the more one got to know him, and one had to know him well to appreciate his worth. Creed seized the first opportunity after the 1939-45 war to drop private practice, not because he disliked it but because he felt it was his duty so that he might better serve his hospital. His conscientiousness resulted in his being grossly overworked during the war, and, though he was not alone in this, there is little doubt that his early and untimely death was largely due to the severe and prolonged strain of E.M.S. sector work in more than one hospital during the enemy onslaughts on London. The profession has lost a distinguished colleague and his friends a shining example of all that a friend should be."

During holiday periods Creed delighted in the country, good hilly country, where he would walk and drink in the beauties of the land. He had more than a passing interest in architecture and a great appreciation of music, especially festival music. For general reading he preferred philosophical and historical works. He had a large circle of friends. Among these he was known and loved for many of the characteristics which in themselves made him less well-known to the world at large than he rightly deserved. His quietness, unobtrusiveness, lack of "swank," honesty, strong reaction to anything false, kindness, generosity and unfailing thought for others, were appreciated. His wife, Stella (*née* Parker) whom he married in 1923, and his two daughters, all of whom have been so well known to Creed's friends, will have the deepest sympathy of those who have been privileged to know them in their own home circle.

LIONEL E. H. WHITBY

### David Arthur Welsh

Born 19th November 1865. Died 13th May 1948

(PLATE CIX)

THE death of David Arthur Welsh, Emeritus Professor of Pathology in the University of Sydney, occurred at Wahroonga, New South Wales, on 13th May 1948. He occupied the chair in Sydney from 1902 to 1936, and his services were of high value in developing the medical school as well as in the teaching of pathology. In research he was a distinguished and enthusiastic worker, and it is especially noteworthy that his most important scientific contributions, starting over fifty years ago, were on such diverse subjects as the parathyroids,

the precipitin reaction and tumour growth. In all of these he maintained a high standard of excellence.

Welsh was born at Montrose in 1865 and received his school education at Arbroath High School, where he won many prizes and was dux of the school. In 1883, equipped with a good general education, he entered upon the arts curriculum at Edinburgh University, and in this he had a distinguished career, excelling especially in the mathematical subjects. He graduated M.A. with first-class honours in mathematics in 1887, gaining the Drummond Scholarship, which was the highest distinction in the subject at that time. In 1888 he started on his medical curriculum, taking also further courses in science concurrently for three years; he graduated B.Sc. in the mathematical sciences in 1890. As a student of medicine Welsh had a course of maintained brilliance; he won many distinctions, and obtained the degrees of M.B., C.M. with first-class honours in 1893. The two years after graduation were chiefly occupied with clinical work. He was a resident physician with Sir Thomas Fraser in the Edinburgh Royal Infirmary, and later with Sir Thomas Clouston in the Royal Edinburgh Hospital for Mental Disorders. This experience was undoubtedly of great service to him in widening his outlook and increasing his interest in the clinical aspects of disease.

In 1895 he was appointed junior university assistant in pathology to Professor Greenfield, and a little later became pathologist to the Edinburgh Royal Hospital for Sick Children, and also one of the tutors in clinical medicine in the Royal Infirmary. At this period he carried out important research work on the parathyroid glands, and a thesis embodying his results gained for him in 1897 the degree of M.D. with first-class honours and the award of a gold medal. In the pathology department of the University, Welsh had Muir as a colleague, and on the appointment of the latter to the chair of pathology in St Andrews in 1898, succeeded to the offices held by him, namely those of lecturer on pathological bacteriology in the University, and pathologist to the Royal Infirmary. Up to that time there had been two part-time pathologists, but in 1901 a new arrangement was made whereby there was a single full-time pathologist who had control of the department, with two assistant pathologists. Welsh was now appointed to the full-time post. The benefits of this arrangement were just becoming evident when he was elected to the newly created chair of pathology in Sydney, taking up his duties there in 1902. He had become M.R.C.P.E. in 1899 and was elected to the Fellowship in 1911.

Welsh's leaving for Sydney was a matter of deep regret to his many colleagues and friends in Edinburgh. His attainments as a pathologist, both in research and in routine work, along with his never failing readiness to give his services, had made him a valuable and prized member of the school, while his attractive personal qualities had won for him the high esteem of all who knew him. Moreover,

he had shown teaching powers of an unusual order. He possessed indeed, in rare combination, the qualifications requisite for success as a professor of his subject.

When Anderson Stuart in physiology and J. T. Wilson in anatomy were joined by Welsh, the Sydney Medical School had three outstanding men to shape its future. These three men, affectionately known to their students as "Andy," "Jummy" and "Taffy," all came from north of the Tweed; they were very different types, but all contributed much to the development of the University of Sydney Medical School in the early years of this century.

On arrival in Sydney in 1902 Welsh found almost virgin soil so far as pathology was concerned, but he settled down to his task with enthusiasm, and in a short time his department established a reputation for excellent teaching and valuable research work.

In addition to his University duties, Welsh acted as honorary pathologist to the Royal Prince Alfred Hospital from 1902 to 1925, and thereafter as honorary consultant pathologist until 1936; he was responsible for all the pathological work of the hospital during the early years after his arrival in Sydney, and in later years his wide experience was always available to help in solving difficult problems. When the radium clinic for the treatment of cancer was opened at the Royal Prince Alfred Hospital in 1928 Welsh, with the help of his son, Dr Arthur M. Welsh, and of Dr Julia Leonie Amphlett, and in collaboration with successive radium registrars, made a study of the radio-sensitivity and radio-resistance of the cancer cell.

Professor Welsh attracted graduates of high calibre to join his staff, among them men and women who have done much to advance pathology in Australia—in universities, in hospitals and in private practice. Great, however, though his influence was in the development of pathology, his influence on students who were to practise medicine in all its branches was still greater. Those who were privileged to study under him will agree that his lectures were full of interest and admirably presented. He laid great stress on practical classes, in which he himself participated, and he took an individual and personal interest in all his students. For those few who worked as his close colleagues he provided a stimulating example because of his outstanding competence in his work, his high intellectual capacity and his cultivated mind. He brought from his homeland to the land of his adoption much that is best in the culture of the Old Country and his influence on medicine in Australia will last long.

He was Dean of the Faculty of Medicine from 1927 to 1929.

Dr A. H. Tebbutt writes (*Medical Journal of Australia*, 3rd July 1948):—"Only those who 'went through' in the first quarter of the century knew 'Taffy' at his best. And let it be recorded in the annals of the Sydney School that for most of that period 'Taffy' was, in contemporary student opinion, the best teacher in the school. Coming from Edinburgh, he was by repute one of Professor Greenfield's







best young men. His speech was doubtless that of the cultured man from Edinburgh, scarcely a Scotch accent, and was as clearly audible as its content was lucid and logical. He had . . . a gentle and graceful wit. His clear but scholarly exposition, his unhurried and orderly progression, and the unexpected little humorous simile all combined to make his lectures both popular with students and not soon forgotten. His attitude to students was sympathetic and understanding. In our time, for example, he found that he could not complete the course in special pathology, so he announced that he would not 'do' the kidney and, to applause, that he would ask no questions on it. . . . Many will remember his autopsies, how simply they were conducted, how he always tried to demonstrate the effects of disease and time on the heart, the great vessels and the kidneys. Welsh was in fact a great teacher of pathology of the old school, dating from Edinburgh at its best."

Welsh's first important investigation was on the parathyroid glands, and was carried out when he was an assistant in Edinburgh University. At that time knowledge of the subject was still in an early stage and many points were obscure. Especially puzzling and in need of investigation was the diversity of the results of thyroidectomy in different classes of animals; sometimes fatal results followed, sometimes only chronic symptoms. The work of various observers suggested the importance of the parathyroids in this connection, but Vassale and Generali were the first to describe fully the acute symptoms with fatal results which followed parathyroidectomy; their experiments were carried out on dogs and cats. Welsh's attention was first drawn to the subject by Ford Robertson, who was an omnivorous reader, and he resolved to repeat the work of the Italian observers. The first requisite was a careful study of the anatomical relations of the glands, and this was duly made. In the experimental work he used cats, and was assisted in the operations by George Carmichael Low, at that time a student of medicine. The writer well remembers the experiments, which were performed in the practical histology class-room after the class work was over. They were carried out with the minimum of equipment, but with high operative skill and scrupulous care as to asepsis, and they were fully controlled by histological methods in relation to the nature of the tissue removed and to the possibility of injuries to the thyroid. He found in confirmation of Vassale and Generali's work that removal of all four parathyroids resulted in the now well recognised acute symptoms—tetany and other muscular phenomena, anorexia etc., followed by death after five days. The removal of three parathyroids might cause some of these symptoms, which however were recovered from; death occasionally occurred when the thyroid was removed at the same time. The removal of two parathyroids had no effect, even when accompanied by thyroidectomy. He further found that the oral administration of ox parathyroid in

large quantities was without effect in ameliorating the symptoms. He considered that the results of these experiments were conclusive as to the symptoms being produced by removal of the parathyroids. The anatomical part of Welsh's work on the parathyroids was published in two papers, and is unusually full. In addition to a review of the whole subject up to that date, there is a useful and detailed account of the gross anatomy of the glands and their relationships in the human subject, while his description of their histology has been accepted as authoritative. The distinctive characteristics of the two chief types of cells are fully described, as are also the variations met with in their arrangement and in certain minor details. Comparison is drawn with other endocrine glands and the resemblance of the parathyroids to the adrenals and anterior pituitary is noted. On the other hand, the dissimilarity between parathyroid and thyroid tissue, even at early stages of development, is insisted on. Welsh's papers on the parathyroids are rich in descriptive detail, and present a valuable picture of the subject as known at that time. They contain the first systematic account of the subject published in this country, and are the greater testimony to his powers in that the work was done while he was engaged in exacting routine duties.

Welsh's next important series of researches were in quite a different field, namely that of serology. They concerned the precipitin reaction, and must have been begun not long after he went to Sydney, the first being published in 1906, at a time when he was engaged in the pioneer work of his chair. Six papers on the subject were published in this country, three of them in the *Proceedings of the Royal Society*, and there were others in Australian journals. Throughout the work he had H. G. Chapman as a collaborator. Knowledge of the subject up to that time is fully presented in Nuttall and Graham-Smith's book on the precipitin reaction, and it may be that this work was the means of stimulating Welsh's interest in the subject. However that may be, he devoted himself for several years to its investigation, and was able to throw fresh light on many important points.

The general view then held was that the precipitin (antibody) on uniting with the antigen precipitated the protein of the latter. It may be recalled that such a view was suggested by Ehrlich's theory of receptors: a precipitin was accounted a receptor of the second order, consisting of a haptophore and an ergophore group, the latter being effective in producing the observed result. Welsh and Chapman showed that this was far from being the case—that in fact the antibody developed in the process of immunisation was to be regarded rather as the precipitable substance than as the precipitant. In such matters it is always difficult to speak of priority, but there is no question as to the original nature of this fundamental observation of Welsh and Chapman. It may be mentioned that Welsh gave a practical demonstration of their results to the Pathological Club of Sydney in 1905. These were substantiated by exact measurement in various ways.

For example, if an interaction is carried out with a small amount of the homologous protein, resulting in massive precipitation, and the supernatant fluid is drawn off, the latter is effective in again producing a precipitate with fresh anti-serum ; and this may be repeated several times. They did not maintain that the reacting substance in the homologous serum is not at all diminished, but they found that it was insensibly exhausted even after repeated interactions. The total amount of protein in the homologous serum is in fact a mere fraction of the mass of precipitate which may be formed by the interaction. In a later paper this was shown by actual weighing of the dry material. The writers suggested that the antigen may exert a catalytic action on the precipitable substance whereby a molecular rearrangement is induced and the substance is thrown out of solution. In accordance with their view it was found that the total amount of protein in the blood serum of an animal increased in the process of development of precipitin.

Other papers are concerned with questions which naturally arose in connection with the authors' conception of the process of precipitation. Two papers deal with the phenomena of inactivation and inhibition produced by heating, the former being a state in which the precipitating (or rather the precipitable) property of an anti-serum is lost, the latter the property of interfering with precipitation when added to fresh anti-serum along with homologous protein. It was shown that these two properties of serum are separable phenomena : for example serum becomes inactive at 72° C., but not inhibitory till 75° C. The anti-serum may also become naturally inactive on being dried, without becoming inhibitory. The inhibitory serum, however, has another property, namely that of dissolving in a specific way the precipitate formed by an interaction ; and inhibitory and dissolving powers were found to be inseparable. They concluded from their results that inhibitory serum acts on neither antigen nor antibody alone but on the product of their combination, and that an anti-serum is inhibitory in virtue of its powers of dissolving precipitate. They considered that the phenomena observed could not be explained on the hypothesis that they were due to "precipitoids" formed by heating. The whole question will be found to be very fully discussed in a paper in this *Journal* (1908-09, xiii, 206). Welsh and Chapman also applied their chief conception to the standardisation of precipitating anti-sera and to the differentiation of group and specific precipitins, etc. All their work is characterised by detailed analysis and full controls and may be regarded as a noteworthy contribution to the subject.

Many other subjects engaged Welsh's attention during his long and distinguished tenure of the Sydney chair. Alone and in collaboration with colleagues he published original contributions on hæmogregarines and other blood parasites of native Australian animals, snake venoms, hydatid disease, actinomycosis, X-ray necrosis (which

must have had a particular personal interest for him), and mental deficiency. In the later years of his active period, between 1929 and 1938, he published a large number of papers on various aspects of neoplasia, many of them in the *Journal of the Cancer Research Committee of the University of Sydney*. Tumour classification was one of these subjects, and he insisted on the need for regional classifications in relation to the certification of causes of death. Contact spread of cancer and progressive carcinogenesis *in situ*, attributable to some form of contagion spreading from cell to cell, and natural defence reactions against cancer were two aspects of the cancer problem which particularly excited his interest.

Of the great esteem in which Welsh was held, in Edinburgh and Sydney alike, we have abundant evidence. He had high ideals of service, and these were carried out in practically every department of his work, both in individual fashion and in co-operation with others. He had, too, an intensely sympathetic interest in his students, and the strong influence which he exerted on the career of many may be readily understood. He had, moreover, two qualities which seem to the writers specially noteworthy. One was his unselfishness in all his relations with his fellows; there was in him no trace of jealousy, and he would rejoice wholeheartedly in the good fortune of others. The other quality was his warm sympathy with those in suffering or distress of any kind. He himself had the misfortune to suffer from a disability which affected him during the greater part of his professorial life. More than thirty years before his death he had X-ray treatment for an affection of the feet, the treatment being carried out both in this country and in Australia. There was definite overdosage and the usual results followed—epithelial thickening and fissuring of the soles of the feet, etc. Later, intractable ulceration followed, leading to progressive incapacity. His powers of walking were impaired, and he was cut off from outdoor recreations such as golf, in which he had previously taken great pleasure. Ultimately, about four years before his death, epithelioma developed in one foot, and amputation of the leg had to be performed; this was unfortunately followed by much pain.

After retirement from his chair, he took up the cause of discharged soldiers and sailors suffering from disabilities of various kinds, going into their cases from the pathological point of view. In this way he succeeded in getting better conditions for many men. This beneficent work was carried on even when he was in a state of suffering—in fact until it was physically impossible for him to continue. Ultimately there was evidence of severe arteriosclerosis leading to coronary thrombosis and retinal changes with impairment of vision. All these trials were borne with great heroism and cheerfulness of spirit, and he maintained to the end his compassionate interest in the well-being of others.

Welsh, in 1900, married Elizabeth Muir, eldest daughter of the

Rev. Robert Muir, M.A., Allars Church, Hawick. They had one son, Arthur Muir Welsh, who entered the medical profession and for a time took part in the teaching of pathology and in research in the University of Sydney.

We are indebted to Dr Mervyn Archdall, editor of the *Medical Journal of Australia*, for permission to quote from the obituary notice which appeared in that *Journal* on 3rd July 1948. A full bibliography of Professor Welsh's published work appears in the same issue.

ROBERT MUIR  
KEITH INGLIS



## BOOKS RECEIVED

### Bone marrow biopsy : hæmatology in the light of sternal puncture

By S. J. LEITNER. English translation revised and edited by C. J. C. BRITTON and E. NEUMARK. 1949. London: J. & A. Churchill, Ltd. Pp. xi and 434; 7 plates (6 in colour) and 188 text figs. 42s.

When a lengthy monograph, cast in the classic mould of continental scholarship, is translated from the German, revised, and expanded by the addition of many references to modern Anglo-American work, the result is likely to be a volume of great erudition but singular indigestibility. So it is with the present volume. Amongst a welter of quoted opinions and references, the diligent reader will find original observations, comments, interpretations and judgments which are of real value, but so much effort has been expended in producing what the translators hope will be "an adequate and reliable reference book" that the volume fails to be what the author wished, a book "mainly intended for practical clinical use."

Its scope is best described by the sub-title, for, although almost all the illustrations are of marrow tissue and cells, much of the text and many of the diagrams are concerned with peripheral blood changes, and the subject matter, apart from the introductory chapters on technique and on the morphology of marrow cells, is systematically presented as in a standard textbook of hæmatology. The documentation is tremendous. The tabulated references at the end of one long chapter on disorders of erythropoiesis number 963—unless the reviewer has lost count somewhere, and a short chapter of barely 20 pages on disorders of thrombocytopoiesis concludes with a list of 156 references, about half of which date from 1939 onwards.

The industry and enthusiasm of the collaborators in this encyclopædic work are matched by the industry and enthusiasm of certain sternal puncturers whose efforts are faithfully and too often uncritically recorded here. Thus we learn, for what it is worth, that "Schretzenmayr (1938) found a marked myeloid reaction and reticulum cell proliferation" in the marrow in smallpox and that Tanahasi was able to advise amputation for gas-gangrene on the finding of "large, round or ovoid, monocytoid cells" in the sternal marrow, since these cells were found in the marrow of limbs amputated from patients whose disease "took a favourable turn." That such a pronouncement as the last should be recorded without comment is one of the results of subjugating thought to the card-index when composing a book.

The text is much interrupted by long lists of authors and dates. This is perhaps inevitable in a book of this kind, but what is avoidable, if only sufficient vigilance is exercised, is the inclusion of useless and indeed almost meaningless statements, *e.g.* (p. 349) "Atypical cases of Hodgkin's disease, such as those of Schultz, Wermbter and Puhl (1924) and Sachs and Wohlwill (1927), are at least allied to the reticulo-endothelioses, in the opinion of Doan and Wiseman (1934), Cionini and Rotta (1934) and Hittmair (1942)." This pregnant sentence serves also as an example of the style of writing in the worst parts of the book, a style which soon befogs the mind of even the most determined reader.

The technical sections in the early chapters are good, and the careful description of the morphology of the marrow cells is illustrated by fine



photomicrographs, those in the chapter on the study of mitosis being of particular excellence. There are important and original observations on post-mortem changes in the marrow, and the reviewer is glad to see the forcible statement that "sternal puncture half an hour after death is already too late to give a result of any value." The terminology used is acceptable. Thus "erythroblast" is "a collective denomination for all nucleated red cells," and "the pathological developmental series of megablasts" is separated from normal erythropoiesis. The plasma cell is clearly one of Leitner's hobby-horses. Not content with a plasmoblast, a proplasmocyte and a plasmocyte, he distinguishes a "semi-mature plasmocyte," and there is much information about cytoplasmic inclusions and vacuoles in these cells and their relation to protein production. The six colour plates are clear and instructive. With a few exceptions, the black and white photomicrographs are well chosen and well reproduced and testify to the author's wide practical experience and to his powers of detailed observation.

This book deserves a place in the libraries and on the shelves of hæmatological scholars. It would be ornamental rather than useful on the working bench, but it can be strongly recommended to anyone who contemplates writing a paper on any aspect of marrow biopsy.

#### **An atlas of bone-marrow pathology**

By M. C. G. ISRAËLS. 1948. London: William Heinemann Medical Books Ltd. Pp. x and 79; 12 plates in colour and 3 text figs. 30s.

This small book is both an atlas and a terse and authoritative description of the technique of sternal puncture and of the normal and pathological marrow cells as seen in smear preparations. It contains only forty-eight pages of text, a short bibliography and a dozen excellent coloured plates. One could wish that the publishers had had sufficient faith in its success to have issued it at a more modest price, but the purchaser will realise that the extra few shillings he has to pay are justified by the great care which has been taken with the illustrations. Seven plates are occupied by paintings of nearly two hundred individual cells. The remaining plates illustrate composite groups of cells representing twenty marrow smears, normal or pathological. In tone, colour and perfect register these plates do real justice to the skill of the artist, Miss Davison, and are amongst the very best available in hæmatological literature. Some of the cells appear small, but they are all drawn to the same scale and their size is actually that which one is accustomed to see with the magnifications usually employed.

The text comprises a short description of technique followed by twelve pages on the morphology of marrow cells. There follow a description of the normal marrow smear and of the myelogram in disease, a brief account of the bone marrow in childhood and a useful chapter containing tables comparing the cellular pattern of the marrow in various disorders. Israëls has simplified the terminology, and the dogmatic stand he has taken on many points which have hitherto been too wordily debated will meet with general approval. The reviewer differs from Israëls in preferring to tap the body of the sternum rather than the manubrium and in separating band-form neutrophils from metamyelocytes, but these are only differences in individual practice. The principles of marrow examination are most excellently set out in this book. In the Manchester clinic a "qualitative assessment" (not "assessment," p. 27) is made in all except difficult or research cases. This saves the expenditure of time on the differential

count and excuses the clinician from ploughing through a long numerical report. This is admirable when the laboratory work is undertaken by an experienced hæmatologist, but if the beginner takes this short cut he runs the double risk of issuing totally misleading reports and of never really learning his way about a marrow preparation. This point might be emphasised in future editions.

The book is strongly recommended. It should be at the side of all pathologists who only undertake the occasional sternal marrow examination. The experienced hæmatologist may find it too brief but he will not be displeased by anything it contains. It is emphatically the book to hand to every embryo clinical pathologist as soon as he begins to study marrow-biopsy material.

#### Bacterial and mycotic infections of man

Edited by REX J. DUBOS. 1948. Philadelphia, London, Montreal : J. B. Lippincott Co. Pp. xiii and 785 ; 97 text figs. and 3 colour plates. \$5.

This handsome book from America deserves a warm welcome. Its 37 chapters are the work of 34 contributors, among whom are names familiar to British bacteriologists, such as Dubos, the editor, T. Francis Jr., Janeway, Maxcy, Murray, Nungester, Sabin, and Swift. The object of the book is to describe, principally for the medical student, the bacteria, moulds and actinomycetes pathogenic for man, and where possible to combine these descriptions with a brief account of the pathology, clinical features and epidemiology of the resulting infections. The material is well and thoughtfully arranged and the student will find the information he needs, both for examination answers and for clinical practice, besides a great deal to stimulate his wider interest in the fundamental problems of biology presented by the phenomena of host-parasite relationships. All this is as it should be.

Bergey's outline classification is given in full although it is admitted that this has not remained unchallenged. Even within the book the contradictions of Bergey's system are apparent, since Blair, who writes the chapter on staphylococci, rightly believes that the term staphylococcus will persist and uses it throughout the discussion, although, illogically, he feels obliged to refer to the type species in Bergey's fashion as *Micrococcus pyogenes* var. *aureus*. It is an agreeable feature of the book that, in spite of some astonishing omissions, there is a higher proportion of references to British work than in many American publications.

It is good to discover that bacteriological thinking on both sides of the Atlantic has points of common sympathy. For example, there is a pleasantly acid reference on page 698 to a theory advanced by "certain speculative epidemiologists." It is surprising, however, to read on the same page that the studies of Schneider and Webster on the effect of diet on response to infection showed that resistance-promoting food elements were of "very limited importance" in influencing the results of experimental salmonella infections in mice. This is not a sound interpretation of the authors quoted, of Schneider's later work, or of the results reported by the experimental epidemiologists from the London School of Hygiene in 1937 and 1938.

Among minor irritations it may be permissible to complain of inconsistent and unwelcome forms of bacterial nomenclature in the list of contents, such as "*Mycobacterium Tuberculosis*," thus written, and such curious plural forms as "The Salmonella," "The Shigella," "The Pasteurella"

and "The Brucella." No. 14 seems to have been omitted from the serially numbered charts. But taken all over, the book is as good as it looks and must certainly receive an honoured place in any good medical library. Its modest price is made possible by a grant from the National Foundation for Infantile Paralysis.

### Heredity in breast cancer

By OLUF JACOBSEN. 1946. Copenhagen; Arnold Busck: London; H. K. Lewis & Co. Pp. 306; 209 text figs. 18s.

The handbook describes an inquiry which has been very carefully undertaken and has followed the same lines as that of Wassink. Jacobsen, however, has improved on Wassink's inquiry, in that he has collected the cancer history of a control series based on non-cancer cases.

Two hundred families of proven cases of breast cancer (termed the probands) from the files of the Danish Cancer Registry were studied so as to ascertain the incidence of cancer of all sites in these families. As controls a series of families of 200 patients of corresponding age groups, who were *not themselves suffering from breast cancer, from the surgical wards of Copenhagen hospitals*, were similarly studied and the results compared with those of the probands. The probands and the control cases were closely questioned as to the fate of their relatives and, where possible, the medical histories were followed up by searching the parish and hospital records. Inexplicably, of the 200 chosen controls 90 were males, which surely must rob the results of some of their value. The author is himself very dissatisfied with his controls, rightly pointing out that in these families cancer is not the subject of such interest as in the families of the probands. In consequence, their histories are probably not as accurate, since the results have probably given too low a cancer incidence. Certainly, looking at the tables setting out the incidence of cancer in the two groups, it would seem that amongst the relatives of the probands the incidence of cancer of all sites, and particularly of the breast, is considerably higher than in those of the control series. The author sees in these results "a clear indication of hereditary predisposition being the chief factor in the development of cancer." But in an inquiry of this nature there is an unavoidable weakness in that much of the evidence depends on the accuracy of the results of questioning, and that there will be less accuracy, as the author himself admits, in the control series.

### Methoden der pathologischen Histologie

By FRÉDÉRIC ROULET. 1948. Vienna: Springer-Verlag. Pp. xi and 567; 20 text figs. Swiss fr. 63 (bound Swiss fr. 66).

Professor Roulet's book follows the general lines of Schmorl's well-known work "Die pathologisch-histologischen Untersuchungsmethoden," a new edition of which it is designed to replace, and contains full and detailed instructions for the use of many relatively new as well as of old and well-known methods and stains. An early chapter contains advice on the use of the microscope, on the cleaning of slides and other apparatus and on the storing of histological preparations. There are sections on phase-contrast and fluorescence microscopy. The directions for the dissection and fixation of various organs and tissues are helpful and practical. Several pages, with useful diagrams, describe the way in which the heart should be dissected and blocks of muscle excised for the study of lesions

of the auriculo-ventricular bundle. A method is described of dissection of the petrous bone for the preparation of material for the microscopical study of the middle and internal ear. For English readers, who have easy access to English and American journals, the omission of a certain number of recently introduced methods is relatively unimportant. On the other hand, in these days of specialisation, the chapters on the preparation and staining of blood films and of films for the demonstration of bacteria, including a list of Gram-positive and Gram-negative bacteria, may appear redundant in a work on morbid histological methods.

This is a valuable work of reference which should find a place in every pathological laboratory. Its special merit lies in the details given of the preparation of the necessary stains and reagents as well as of the conduct of the methods described. Such details are evidence of the author's personal experience and practical familiarity with histological technique.

### The thyroid and its diseases

By J. H. MEANS. Second edition, 1948. Philadelphia, London, Montreal : J. B. Lippincott Co. Pp. xviii and 571 ; 13 plates and 50 text figs. \$12.

This is a book for the practising clinician. The first edition appeared some eleven years ago, and in this—the second—the material has been extensively revised and brought up to date, particularly in the fields of therapy. R. W. Rawson is responsible for the chapters on pathology and tumours of the thyroid, while the surgery is in the hands of Oliver Cope. The book admirably co-ordinates the work of a progressive team of physicians, surgeons and research workers of world-wide repute, and everywhere is to be found a sane, balanced opinion and a critical appraisal of all progressive work in the field of diseases of the thyroid, much of this by men attracted to and working in collaboration with the author in the Thyroid Clinic at the Massachusetts General Hospital. The clinician will find almost every problem of the diseases of the thyroid touched upon and usually fully and critically discussed. One may mention the adoption of the measurement of epithelial cell height as a measure of the functional activity of the thyroid gland, of tissue-culture technique for measuring thyrotropic and anti-thyrotropic factors, the uses and limitations of anti-thyroid drugs, the present position of iodine and surgery and the use of radio-active iodine. The fundamental work on iodine therapy still occupies a prominent place in the book but not at the expense of other topics. There is a full bibliography. The book is a triumph of friendly co-operation, co-ordination and progress.

### A catalogue of insecticides and fungicides. Vol. I, Chemical insecticides

By DONALD E. H. FREAR. 1947. Waltham, Mass. : The Chronica Botanica Co. ; London : William Dawson and Sons, Ltd. Pp. xii and 204. \$6.50.

### A catalogue of insecticides and fungicides. Vol. II, Chemical fungicides and plant insecticides

By DONALD E. H. FREAR. 1948. Waltham, Mass. : The Chronica Botanica Co. ; London : William Dawson and Sons, Ltd. Pp. xii and 154. \$5.50.

During recent years a great deal of research has been directed towards the finding of new materials for insect and fungoid pests. Since the discovery of the active principles of pyrethrum and derris, the search for new and better materials has been intensified and has led to the discovery

of many promising and important new compounds. Many of the commonly used materials are not highly efficient and most of them which have a high toxicity to the lower forms of life are also toxic to both man and the higher animals. At the same time it has become apparent that new strains of pests are arising which have become highly resistant to the older forms of treatment, so that new pest-control substances are needed. The work has been pursued so actively that the literature upon the subject is now both voluminous and widely scattered and to meet the needs of current research a comprehensive survey of the entire field is urgently required.

With this end in view, the author has compiled this catalogue in which are presented data obtained from published and unpublished reports concerning some 10,000 compounds tested against insects and fungi before 1944.

Vol. I deals with chemical insecticides in which the chemical name and formula of each substance is followed by the insects against which it has been tested, and where possible the concentration at which it was employed, the bibliographical references including references to patents. There are also reference and author indexes and a list of patent numbers arranged by countries.

Vol. II deals with fungicides of both synthetic and plant origin and insecticides obtained from plants, again with an extensive bibliography and list of patents and an index of chemical compounds covering both volumes.

The synthetic compounds are arranged according to a new numerical coding system, as briefly explained in the introduction. The whole point of this coding system appears vague and unnecessary. To the chemist it makes the classification very involved and to the biologist it is unintelligible. Until structure can be related to toxicity, it is advisable to retain the system of chemical classification founded on groups and derivatives thereof, which is familiar to all chemists and most biologists. However, this difficulty is easily overcome by the use of the index of chemical compounds at the end of vol. II.

The arrangement of the catalogue is primarily of interest to the chemist and will be of great value in the search for new insecticides and fungicides. Its value would be enhanced by the addition of a biological index giving references to materials used against each pest.

#### **A textbook of clinical pathology**

Edited by FRANCIS P. PARKER. Third edition, 1948. Baltimore: The Williams and Wilkins Co. (British agents Baillière, Tindall and Cox.) Pp. xx and 1023; 44 plates (33 in colour) and 229 text figs. 50s.

The first (1938) edition of this now well-established book was edited by Dr R. R. Kracke, the second (1940) by Dr Kracke and Dr Parker together: the present edition was prepared by Dr Parker alone and had been brought up to page proof just before his untimely death at the end of 1947. Each of the later editions has added some 200 pages to its forerunner and the book is now as large as a single volume ought to be.

Excellent monographs devoted to special branches of clinical pathology are available and British pathologists generally prefer to use these, many of which are of native origin and fully cover the standard practice in this country. It can hardly be stated, therefore, that Parker's textbook is indispensable. Indeed it suffers from the seemingly inevitable fault of comprehensive manuals embodying the work of several contributors—that it is neither advanced enough for the trained clinical pathologist nor

simple enough to serve as a primer for the beginner. Nevertheless the book contains so much of value and interest and is, generally speaking, so clearly written and illustrated that it is bound to prove a useful addition to the library of any laboratory.

Stress is laid throughout the book on the indications for the various laboratory examinations and on the interpretation of results. Technical methods are described in most sections with such clarity of detail that the procedures can easily be carried out from these instructions, and space has been economised by quoting usually one method only for securing the desired result. This is no bad thing, and the methods described have obviously been selected with care. Newer techniques have been substituted for older ones where necessary, and although some of the methods may not be those in current use on this side of the Atlantic, it is useful to have this summary of modern American practice. Dr Parker's chapter on clinical blood chemistry is a fine example of this mode of presentation and should prove of the greatest value to those whose main interests are not chemical but who are occasionally called upon to make the less familiar estimations.

The standard of the different chapters varies. The opening chapter on general hæmatology by L. W. Diggs is excellent; it is clearly written and contains many simple practical observations. Its 94 pages can be strongly recommended for any technician to read. The later chapters on hæmatology are less uniformly good and are not free from error. One cannot allow fig. 55, for example, to pass without comment since it includes a drawing of a hæmatocrit tube with cells packed down to 63 per cent. and labelled "Macrocytic anæmia." Again there is some confusion as to what is meant by a megaloblast. But the chapter on the tests used in hæmorrhagic diseases, also by Diggs, is very good, as is that on the blood groups by L. Davidsohn. This provides an excellent and practical description of its subject, marred only by the use of Wiener's classification of the Rh factor instead of the more logical British system. These chapters are obviously addressed to the more experienced pathologist, as are those on the assay of vitamins and hormones, the diagnosis of venereal lesions and the serological tests for syphilis, including the detailed technique of three complement-fixation and five flocculation tests.

The work falls short of requirements in regard to bacteriology, for although some methods and culture media are briefly described and the diagnosis of rabies occupies ten pages, it would be quite impossible from the instructions provided to conduct a satisfactory investigation of a suspected case of either diphtheria or dysentery or even to deal properly with a throat swab.

Each chapter contains a selected and up-to-date bibliography, referring almost exclusively to American publications. There are many good points about this volume, and some sections are certain to please everyone. Errors and misprints are few, and the production is admirable.

#### The Rh blood groups and their clinical effects

By P. L. MOLLISON, A. E. MOURANT and R. R. RACE, Medical Research Council Memorandum no. 19. 1948. London: H.M. Stationery Office. Pp. 74; 3 text figs. 1s. 6d.

"It is inevitable that a discovery which is so intellectually satisfying and so practically effective should gather round it a bulky literature, and one that will grow as the researches enter the fields of genetics and ethnology. The Medical Research Council, realising that there is an urgent need for an authoritative summary of present knowledge on this subject, believe

that this memorandum by Dr Mollison, Dr Mourant and Dr Race will be widely welcomed." So ends the preface to this excellent monograph, and all who have attempted to follow the literature since the discovery of the Rhesus factor will heartily concur in these remarks. Landsteiner and Wiener announced the discovery of the Rhesus factor in 1940, and almost immediately its significance in intra-group hæmolytic transfusion reactions and hæmolytic disease of the new-born became known. The effect of these announcements can only be likened to a veritable medical "Klondike," with so many workers anxious to "stake a claim." This has resulted in a continuous stream of contributions on all aspects of the subject, some significant, others not. The resulting confusion of thought, to which must be added controversy in regard to terminology, has rendered the subject almost unintelligible to any but those actively engaged in the work. After realising the need for an authoritative review it was only to be expected that the Medical Research Council should call on the present authors, who have already made such outstanding contributions in this country, to fulfil this task.

For convenience the memorandum is divided into three sections:—(I) The Rhesus groups, (II) Clinical Considerations, and (III) Rh Testing. Race, after a short historical introduction, explains the simple division of the Rh groups into Rh-positive and Rh-negative. This explanation is brilliantly done and no serious reader can fail to understand and appreciate its significance. He then proceeds to a more complete description of the Rh groups, the different Rh antibodies, the effect of the ABO blood groups on the formation of anti-Rh and concludes with a short but lucid explanation of Fisher's genetical basis of the Rh groups.

Mollison deals with the second section and in a precise and lucid style explains the effects of iso-immunisation. He then discusses the diagnosis and treatment of hæmolytic disease and concludes with some sound suggestions for transfusion technique. Such controversial issues as termination of pregnancy and breast feeding are admirably dealt with, although one feels that a worker of Mollison's ability and experience could have afforded to be more dogmatic. Although this section is mostly concerned with iso-immunisation and its sequelæ, all clinicians who have recourse to blood-transfusion therapy will profit by its study. This clear exposition is probably the best of its kind to appear in print so far, and should remain the standard for clinical practice for some time to come.

Laboratory workers will welcome Mourant's clear account of the many aspects of Rh testing, the section on the testing of cells and serum being particularly good. Pathologists who carry out large numbers of routine tests will be surprised, however, by the lack of emphasis placed on the necessity for adequate controls, and on the instability of immune sera. In the list of equipment necessary for carrying out these tests no mention is made of a centrifuge.

Altogether this is a most excellent monograph and the authors and the Medical Research Council are alike to be congratulated on fulfilling a long-felt want.

#### **Die Immunitätsforschung, vol. III, Die Antigene**

By R. DOERR. 1948. Vienna: Springer-Verlag. Pp. vii and 375; 3 text figs. Swiss fr. 38.

This volume contains much compressed information about antigens. It is essentially a work of reference for the specialist in serology who is able to make use of the concise summaries of and the references to important and recently published observations. It is not a book for beginners and





the author in the review of an extensive literature. We have the advantage of his labour in this up-to-date and comprehensive textbook especially intended for the dental student and practitioner. It should meet their requirements more than adequately, and will be useful also to the medical practitioner and to those engaged on special investigations in this field.

#### Laboratory diagnosis of protozoan diseases

By CHARLES FRANKLIN CRAIG. Second edition, 1948. London: Henry Kimpton. Pp. 384; 7 colour plates and 56 text figs. 27s. 6d.

The first edition of this book was published in 1942 and was at once accepted as authoritative. It proved of great practical value to all workers, both at home and abroad, who were engaged in diagnosing the vastly increased number of cases of protozoal infections resulting from wartime conditions. Col. Craig gives two reasons for the appearance of the second edition, namely the exhaustion of the first, and the need for including new material, particularly in regard to those two preponderatingly important tropical diseases, malaria and amœbic dysentery. The importance of diagnosing these diseases with the minimum of delay and the maximum of certainty is all-important, not only for the sake of the patient, but to prevent the spread of infections introduced by large numbers of men returning from overseas. Very properly, therefore, some two-thirds of the new edition are devoted to their consideration. In the case of malaria, although many serological and biochemical methods of diagnosis are discussed, the fact remains that the only satisfactory method of diagnosing malaria is the finding of the parasite. Cultural methods employed for this purpose have so far proved unsatisfactory, and reliance must still be placed on the search of thin and thick films taken from the peripheral blood, the greatest advance in rapid diagnosis having followed the introduction of improved methods of preparing and staining thick blood films. As regards the diagnosis of amœbic dysentery, the situation is somewhat different. It is generally agreed that when examining the faeces for *histolytica* cysts only some 1 in 5 infections will be detected by a single direct smear examination, and that this proportion of positives will be considerably increased by the employment of one of the recognised concentration methods. Of these, the zinc sulphate centrifugal flotation method is recommended. On the other hand, whereas British workers seldom use the complement-fixation test as a method of diagnosing amœbic infections, Col. Craig regards it as a valuable diagnostic aid, although he is careful to point out that it should never replace the stool examination.

The remaining one-third of the book deals with methods for diagnosing leishmaniasis, trypanosomiasis, balantidiasis and coccidiosis; as regards this last-named infection, it is pointed out that there is no worth-while evidence that *Isospora hominis* is in any way pathogenic to the human host. On the contrary there is reason to think that, as in the case of *E. sardina* and *E. clupearum*, it may be only a passenger through the human intestine. In each of these infections the various methods for obtaining, preparing and examining material are discussed, as is also the comparative value of different methods of diagnosis. It might be argued that the author presents too many alternatives, but this book is intended for the experienced worker, not for the novice, and the advantages and disadvantages of each method are carefully considered. The reviewer has only two suggestions to offer: if, as appears probable, there is a call for a third edition, this might include a reference to the value of examining the passed placenta for evidence of malaria and, in the case of African trypanosomiasis, the

value of examining the site of the infective bite—the chancre—for the presence of trypanosomes, before these have had time to invade the lymph- or blood-stream.

The work is finely produced and well illustrated, the quality of the binding, paper and printing being exceptionally good. In view of the numerous illustrations, mainly from photographs, and the fine colour plates, the price charged—27s. 6d.—is not unduly high.

#### Essentials of public health

By WILLIAM P. SHEPARD. 1948. Philadelphia, London, Montreal: J. B. Lippincott Co. Pp. xviii and 600; 18 plates, 2 text figs. and 29 charts. \$5.

This book, written in collaboration with C. E. Smith, R. R. Beard and L. B. Reynolds, is intended for practising physicians, not laboratory or public health workers, and is thus unlikely to be of particular interest to readers of this *Journal*, while the terse style will probably jar on the average British reader. Some of the material in the book gives the impression that one of the purposes of its publication is to convince American physicians what a wonderful place the United States of America is, even if, in order to do so, it requires such casual treatment of the vital statistics as a comparison between the expectation of life in the U.S.A. for 1932-34 and, for example, that in New Zealand for 1921-22, or British India for 1921-30. It seems extraordinary that in a book published in 1948 the most recent figures on maternal mortality quoted relate to 1930. As many significant changes have occurred in maternal mortality since that date the relevant text is of little value.

There are 16 pages of photographs of large sewage disposal plants which can have little interest to the practising physician, whereas the English and Danish work on scabies since 1941 does not seem to have produced any dollar return in the shape of accurate facts on the incubation period and transmission of this disease.

#### Textbook of public health

By W. M. FRAZER and C. O. STALLYBRASS. 12th ed., 1948. Edinburgh: E. & S. Livingstone, Ltd. Pp. xi and 571; 21 plates (1 in colour) and 63 text figs. (1 in colour). 30s.

It must be disheartening for the writers of works on public health to see a new act such as the National Health Service Act, 1946, on the statute book. The main provisions can be expounded in quite a few lines in a new chapter, but, unfortunately, the effects are so widespread that nearly all other public health legislation is modified in some way, thus making the text out of date. In this, the 12th edition of Frazer and Stallybrass, the authors claim in their preface to have produced an up-to-date book, but, in fact, it is merely the 11th or an even earlier edition with a few footnotes and explanatory paragraphs added and a chapter on the new act. The result is a textbook likely to be misleading to the student who studies the book as a whole, and one which will almost certainly prove dangerous to anyone using it for reference purposes.

For instance, on pp. 19 and 20 the duties of Local Authorities are given as they were before 5th July 1948 and, in the section on occupational hygiene, the Workmen's Compensation Act is described, although this, of course, has been repealed. In the section on tuberculosis, financial aid for tuberculous patients is described under the Local Authorities Scheme, whereas it is now part of the ordinary form of National Insurance. Numerous faults of

this kind are not compensated for by the occasional footnotes, which may be many pages away from the text the student is actually reading.

Turning to the more scientific aspects of the book, the section on bacterial food-poisoning is not up to the standard one would expect in a modern textbook in view of the importance of this subject. In dealing with scabies it is stated that disinfection of clothing is usual and the Scabies Order, 1941, is quoted although this has now been repealed.

The writers state that in the case of doubtful diagnosis of smallpox "resort may be had to certain laboratory tests," but these are not described and no indication is given as to their value. The section on diphtheria immunisation savours of having been written ten or fifteen years ago; control of an epidemic by Schick testing and swabbing is not the normal procedure in our present highly immunised population. The treatment of cerebrospinal fever appears to have been written at the time of the introduction of the sulphonamides, while the chapter on streptococcal infections seems to have been written prior to this time and inoculation with toxin is seriously suggested as having a place in the prevention of these diseases. Disinfectant gargles are still advocated for the use of contacts in cases of poliomyelitis. The mortality from anthrax is given as 4 per cent. and treatment does not seem to have advanced beyond the use of serum or excision, no mention being made of sulphonamides or penicillin.

Indigenous malaria is stated to be absent in Britain, which is just not true, and the only reference to mepacrine is most unsatisfactory, located in the hodge-podge paragraphs on chemotherapy and chemo-prophylaxis tucked away in a later part of the book.

It is a pity that a further impression was not taken of the 11th edition rather than producing what in name is a new edition, but is, for all practical purposes, an old one.

### The basis of chemotherapy

By THOMAS S. WORK and ELIZABETH WORK. 1948. Edinburgh: Oliver and Boyd Ltd. Pp. xx and 435: 1 plate and 42 text figs. 26s.

The authors' attitude to their predecessors' work seems to be expressed by the following statements. "The history of chemotherapy during the last half century is largely one of painstaking development by the organic chemist of chance observations made by the experimental pathologist or the microbiologist". "The early successes achieved by chemotherapy tended to obscure the empirical nature of the researches leading to these results. The chief reason for this empiricism was that, until comparatively recently, research was concerned mainly with the action of drugs when injected into infected animals". But the essence of chemotherapy is, as Ehrlich put it, "to cure infected animals". Until this was accomplished there was no chemotherapy; and to dub such work and the consequent biological discoveries of drug-fastness, serum-resistance and chemotherapeutic interference as chance achievements of empirics sounds rather like ingratitude. Without these observations, one wonders what there might have been for biochemists and pharmacologists to discuss in this field. No one should dissent from the dicta that "For chemotherapy to grow as a science, attention must be devoted to the fundamental aspects of drug action. Drug distribution in the host must be understood; the permeability of cells to drugs and the effects of drugs on cell permeability must be elucidated; the altered metabolism and reproduction of pathogen in the presence of drugs must be interpreted in terms of cellular biochemistry. All these problems and more constitute the foundations for a science of

chemotherapy; at the present time we are only beginning to probe their nature". "Our best approach to the foundation is to obtain as clear a picture as possible of the enzymic make-up of living cells, so that, eventually, we can reconstruct in exact chemical terms the series of events, which we call cell metabolism, by which life is carried on". Accordingly, five of the seven chapters, two-thirds of the book, deal with "Cell Metabolism", "Essential Metabolites", "Enzyme Inhibition", "Drug Antagonism", "The Relation of Structure and Activity of Drugs". Thus "The Basis" regards chiefly the biochemical aspects of chemotherapy. While the opinion is expressed that "The theoretical developments . . . begin to provide a rationale for the whole subject", the biochemical foundations are still admittedly frail, as this expression shows—"Since whole groups of enzymes possess certain common characters, such as a common prosthetic group or co-enzyme, it is probable that a drug will affect not a single enzyme but a whole group of related enzymes, some more, others less, essential to life. The problem of reducing drug action to interference with any single enzyme is therefore immense, and the study of intermediary metabolism is likely for the present to do more towards suggesting new types of drugs than towards solving the problem of the mode of action of known drugs". The optimistic conclusion is arrived at that "In chemotherapy the transition state has been reached; theory has overtaken fact and has begun to shape the future. In the process, it has inevitably drawn upon the more mature sciences, physical chemistry, organic chemistry and biochemistry, and in its turn it now has something to offer to those sciences and to pharmacology".

There are about 900 references, nevertheless the authors ask indulgence for their "limited fund of time and energy available after full days at the laboratory bench". This exposition will prove of great use to all interested in the problems of chemotherapy if they bear in mind that the scope is distinctly eclectic, that in-vitro approaches to solving chemotherapeutic problems so far are only tentative and that the biological defence mechanisms of the host often play a significant part in determining the therapeutic result.

C. H. B.

#### List of species maintained in the national collection of type cultures

Medical Research Council Memorandum no. 21. 1948. London: H.M. Stationery Office. Pp. 17. 9d.

A new edition of the Catalogue of the National Collection of Type Cultures is expected to take several years to prepare, and in the meantime this "List" gives the species available and the number of each in the Collection. The foreword gives advice to users of the Collection on a number of practical details. For example, the names used are those given to the organisms by the depositors and the Collection gives no guarantee that they have been correctly named. During the war many strains became contaminated and the Curator will appreciate being told by users if the cultures supplied have suffered in this way.

The terminology used in the list is of interest in view of the many arguments, likely to be continuous, about the merits and failings of different systems of bacterial nomenclature. In our opinion, the usages in the list represent a sensible view that will be acceptable to the majority of British medical bacteriologists.



# INDEX OF SUBJECTS

	PAGE		PAGE
ABSCCESS CAVITIES in lung: lining of healed but persistent abscess cavities in lung with ciliated columnar epithelium . . . . .	259	BLOOD: anæmia in leukaemia . . . . .	63
ACHOLURIC JAUNDICE, red-cell fragility in . . . . .	315	erythrocyte fragility, quantitative description of . . . . .	315
ACID-FAST ORGANISMS: a new myco- bacterial infection in man . . . . .	93	fluidity of, <i>post mortem</i> . . . . .	413
ACTINOMYCOSIS in dogs . . . . .	563	hæmolytic in fresh blood films . . . . .	140
ADAMANTINOMA of jaw with pulmonary metastases . . . . .	193	siderocyte count in blood of normal and premature infants . . . . .	342
ADDISON'S DISEASE due to congenital hypoplasia of adrenals . . . . .	323	BODY FLUIDS: their redistribution in dysenteric toxæmia . . . . .	609
ADRENAL HYPOPLASIA as a cause of infantile Addison's disease . . . . .	323	BRAIN, cysticercosis of . . . . .	505
AGGLUTINATION of <i>Bact. coli</i> var. <i>neapo-</i> <i>litanum</i> in summer diarrhoea . . . . .	395	BRONCHIECTASIS in laboratory rats . . . . .	520
of coliform bacilli by normal rabbit sera . . . . .	307	BRUCELLA, a simple method for testing dye sensitivity of . . . . .	328
AGRANULOCYTOSIS, massive colony formation of <i>Bact. friedländeri</i> in liver in . . . . .	51	BULLET causing pulmonary embolism . . . . .	205
ANÆMIA in leukaemia . . . . .	63	CARCINOGENESIS, EXPERIMENTAL: effect of castration on induction of mammary tumours by oestrogen in male Strong A strain mice . . . . .	517
ANEURYSM of aorta, dissecting, experi- mental production of . . . . .	43	hepatotoxic action of <i>p</i> -dimethyl- aminoazobenzene . . . . .	461
of both pulmonary arteries . . . . .	379	CASTRATION: its effect on induction of mammary tumours by oestrogen in male mice of Strong A strain . . . . .	517
ANTHRAX in a laboratory worker . . . . .	253	its effect on the action of some barbiturates . . . . .	239
ANTIBODY PRODUCTION in cold-blooded vertebrates, effect of temperature on . . . . .	87	CATALASE production by Gram-positive cocci . . . . .	135
ANTIBODY RESPONSE in radiation lymphopenia and the reticuloses . . . . .	123	CHEMOTHERAPY of <i>Trypanosoma con-</i> <i>golense</i> infection with phenan- thridinium compounds . . . . .	336
ANTIGENS of <i>Cl. welchii</i> . . . . .	495	<i>Clostridium welchii</i> , kappa and lambda antigens of . . . . .	495
AORTA, experimental production of dissecting aneurysm of . . . . .	43	CLOTTING of human citrated plasma by Gram-negative organisms . . . . .	247
thrombosis as a factor in the patho- genesis of atherosclerosis of . . . . .	57	COCCI: catalase production by Gram- positive cocci . . . . .	135
ARTERIES: causation of acute arterial necrosis in hypertensive disease . . . . .	357	COCKCHAFER, intestinal flora of . . . . .	510
ARTERIOSCLEROSIS, experimental pul- monary . . . . .	289	COCKROACH, intestinal flora of . . . . .	506
ASTHMA, disseminated focal necrosis with eosinophilia and arteritis in . . . . .	489	CONGENITAL HEART DISEASE: Ebstein's anomaly . . . . .	387
ATHEROSCLEROSIS of aorta: throm- bosis as a factor in its pathogenesis . . . . .	57	trilocular heart . . . . .	379
BACTERIA in human semen . . . . .	131	CONNECTIVE TISSUE, spread of fluids in CORYNEBACTERIA from nose and throat . . . . .	403
<i>Bacterium aerogenes</i> , influence of cultural conditions on morphology of . . . . .	265	<i>Corynebacterium diphtheriæ</i> , bacterial variation and toxin production by mutation in single-cell cultures of . . . . .	595
<i>Bacterium coli</i> , var. <i>neapolitanum</i> in summer diarrhoea . . . . .	395	CYSTICERCOSIS CEREBRI . . . . .	505
non-gas-producing variant induced by growth in glycine . . . . .	521	CYTOLOGY: cytoplasmic basophilia of marrow cells: distribution of nucleic acids . . . . .	1
<i>Bacterium friedländeri</i> : massive colony formation in liver in agranulo- cytosis . . . . .	51	DEGENERATION, PARENCHYMATOUS, mechanism of its production by diphtheria toxin . . . . .	75
BARBITURATES, effect of castration on action of . . . . .	239	DIPHThERIA TOXIN, mechanism of parenchymatous degeneration pro- duced by . . . . .	75

	PAGE		PAGE
DIPHTHEROID BACILLI from nose and throat . . . . .	403	LEUCOCYTES in human semen . . . . .	131
DOG, actinomycosis in . . . . .	563	LEUKAEMIA, anemia in . . . . .	63
DYSENTERY: redistribution of body fluids in dysenteric toxæmia . . . . .	609	LIPOSARCOMA arising in simple lipoma . . . . .	483
EBSTEIN'S ANOMALY of tricuspid valve . . . . .	387	LIVER, glycogen content after poisoning with <i>p</i> -dimethylaminoazobenzene . . . . .	573
ECTROMELLA, pathogenesis of . . . . .	529	LOEFFLER'S SYNDROME . . . . .	489
EMBOLISM of lung by a bullet . . . . .	205	LUNGS: embolism of lung by a bullet . . . . .	205
ENDOCARDITIS, acute parietal, in status asthmaticus . . . . .	621	epithelial lesions in respiratory tract in human influenzal pneumonia . . . . .	429
ENTEROCOCCI: catalase production by Gram-positive cocci: a simple test for differentiating enterococci from micrococci . . . . .	135	lining of healed persistent abscess cavities with ciliated columnar epithelium . . . . .	259
EPIDERMIS, melanin-forming tumours of . . . . .	21	neoprene latex casts of . . . . .	354
FALLOPIAN TUBE granuloma . . . . .	512	LYMPHATICS: factors concerned in intracutaneous injection of cattle . . . . .	137
FIBRINOLYSIS and fluidity of blood <i>post mortem</i> . . . . .	413	LYMPHOPENIA: antibody response in radiation lymphopenia and the reticuloses . . . . .	123
FLAGELLA as organs of locomotion . . . . .	275	MARROW in glandular fever . . . . .	629
FRAGILITY of erythrocyte, quantitative description of . . . . .	315	MARROW BIOPSY in rabbits . . . . .	332
GLANDULAR FEVER, bone marrow in . . . . .	629	MARROW CELLS, cytoplasmic basophilia of . . . . .	1
incidence of pathogenic staphylococci in throat in . . . . .	157	MELANIN-FORMING epidermal tumours . . . . .	21
GLOMERULO-NEPHRITIS: auto-immunisation hypothesis of its pathogenesis . . . . .	211	METAPLASIA, squamous, in prostate in stilbæstrol therapy . . . . .	330
GLYCINE, non-gas-producing variant of <i>Bact. coli</i> induced by growth in . . . . .	521	MONONUCLEOSIS, INFECTIOUS, bone marrow in . . . . .	629
GLYCOGEN content of liver after poisoning with <i>p</i> -dimethylaminoazobenzene . . . . .	573	MOUSE: effect of castration on induction of mammary tumours by œstrogen in male Strong A mice . . . . .	517
its histological demonstration in necropsy material . . . . .	325	tissue changes in mice treated with pentose nucleotides . . . . .	441
GRANULOMA of Fallopian tube . . . . .	512	MOUSE-POX, pathogenesis of . . . . .	629
HÆMOLYSIS in fresh blood films . . . . .	140	MUTATION in single-cell cultures of <i>C. diphtheria</i> . . . . .	369
HEART: bullet into right ventricle . . . . .	205	MYCOBACTERIAL INFECTION in man: a new type . . . . .	93
Ebstein's anomaly of tricuspid valve idiopathic ("isolated") myocarditis in infancy . . . . .	477	MYOCARDITIS, idiopathic ("isolated"), in infancy . . . . .	477
primary sarcoma of . . . . .	344	NECROSIS in asthma, disseminated focal, with eosinophilia and arteritis . . . . .	489
trilocular . . . . .	379	NEPHRITIS: auto-immunisation hypothesis of its pathogenesis . . . . .	211
HYPERTENSION, acute arterial necrosis in . . . . .	357	NOCARDIOSIS in a Bennett's wallaby . . . . .	155
IMMUNITY: immunological studies with group-B streptococci . . . . .	219	NUCLEIC ACIDS in basophilic marrow cells . . . . .	1
INFLUENZAL PNEUMONIA, epithelial lesions in respiratory tract in . . . . .	429	OBITUARY NOTICES— Edward folliott Creed (portrait) . . . . .	635
JAW, adamantinoma of, with pulmonary metastases . . . . .	193	David Arthur Welsh (portrait) . . . . .	638
KAOLIN: its effects on rat lungs . . . . .	435	PENTOSE NUCLEOTIDES, tissue changes in mice treated with . . . . .	441
KIDNEYS: auto-immunisation hypothesis of the pathogenesis of glomerulo-nephritis . . . . .	211	PHENANTHRIDINUM compounds in the treatment of <i>Trypanosoma congolense</i> infection . . . . .	336
		PHOSPHATASE, SERUM ACID, application of formaldehyde to determination of . . . . .	149
		PNEUMONIA, INFLUENZAL, epithelial lesions in respiratory tract in . . . . .	429
		PNEUMONOCOONIOSIS: effects of kaolin on rat lungs . . . . .	435

	PAGE		PAGE
POLAROGRAPHIC ANALYSIS of patho- logical sera . . . . .	152	REVIEWS OF BOOKS—continued	
PROCEEDINGS OF THE SOCIETY . . . . .	147, 353	Submicroscopic morphology of proto- plasm and its derivatives (A. Frey- Wyssling) . . . . .	523
PROSTATE: squamous metaplasia in stilbæstrol therapy . . . . .	330	The leptospiroses (P. H. van Thiel) . . . . .	523
PROTEUS, rapid identification of . . . . .	183	Diagnostic procedures for virus and ricketsial diseases . . . . .	524
urease and other biochemical re- actions in <i>Proteus</i> group . . . . .	171	Gynæcological histology (Josephine Barnes) . . . . .	525
PULMONARY ARTERIOSCLEROSIS, experi- mental . . . . .	289	Biology of pathogenic fungi (Ed. by Walter J. Nickerson) . . . . .	526
RABBIT, marrow biopsy in . . . . .	332	Laboratory technique in biology and medicine, 2nd ed. (E. V. Cowdry) . . . . .	526
RADIATION: antibody response in radiation lymphopenia and the reticulosés . . . . .	123	Oxidation-reduction potentials in bacteriology and biochemistry, 5th ed. (L. F. Hewitt) . . . . .	527
RAT: bronchiectasis in laboratory rats effects of kaolin on lungs . . . . .	520	Practical section cutting and staining (E. C. Clayden) . . . . .	527
glycogen content of liver after poisoning with <i>p</i> -dimethylamino- azobenzene . . . . .	573	Diagnosis in daily practice (Benjamin V. White and Charles F. Geschickter) . . . . .	528
RETICULO-ENDOTHELIAL CELLS: their demonstration in paraffin sections . . . . .	515	Bone marrow biopsy: hæmatology in the light of sternal puncture (S. J. Leitner) . . . . .	647
RETICULOSES, antibody response in . . . . .	123	An atlas of bone-marrow pathology (M. C. G. Israël) . . . . .	648
REVIEWS OF BOOKS—		Bacterial and mycotic infections of man (Ed. by René J. Dubos) . . . . .	649
Lectures on the liver and its diseases (H. P. Himsworth) . . . . .	142	Heredity in breast cancer (Oluf Jacobsen) . . . . .	650
The nature and prevention of the cereal rusts as exemplified in the leaf rust of wheat (K. Starr Chester) . . . . .	142	Methoden der pathologischen Histologie (Frédéric Roulet) . . . . .	650
The UFAW handbook on the care and management of laboratory animals (Ed. by Alastair N. Worden) . . . . .	143	The thyroid and its diseases, 2nd ed. (J. H. Means) . . . . .	651
Symposium on medicolegal problems (Ed. by Samuel A. Levinson) . . . . .	144	A catalogue of insecticides and fungicides; vol. I, Chemical insecticides; vol. II, Chemical fungicides and plant insecticides (Donald E. H. Frear) . . . . .	651
Standard methods of the division of laboratories and research of the New York State department of health, 3rd ed. (A. B. Wadsworth) . . . . .	144	A textbook of clinical pathology, 3rd ed. (Ed. by Francis P. Parker) The Rh blood groups and their clinical effects (P. L. Mollison, A. E. Mourant and R. R. Race) . . . . .	652
Principles of medical statistics, 4th ed. (A. Bradford Hill) . . . . .	145	Die Immunitätsforschung; vol. III, Die Antigene (R. Doerr) . . . . .	653
Gardiner's Handbook of skin diseases, 5th ed. (Revised by John Kinnear) Handbook of practical bacteriology, 8th ed. (T. J. Mackie and J. E. McCartney) . . . . .	145	Oral and dental diseases: ætiology, histopathology, clinical features and treatment (Hubert H. Stones) Laboratory diagnosis of protozoan diseases, 2nd ed. (Charles Franklin Craig) . . . . .	654
The biology of melanomas (Ed. by Roy Waldo Miner, Myron Gordon and Lothar Salin) . . . . .	146	Essentials of public health (William P. Shepard) . . . . .	655
The 1947 year book of pathology and clinical pathology (Ed. by Howard T. Karsner, Herbert Z. Lund and Arthur Hawley Sanford) Textbook of endocrinology (Hans Selye) . . . . .	347	Textbook of public health, 12th ed. (W. M. Frazer and C. O. Stally- brass) . . . . .	657
The digestive tract in roentgenology (Jacob Buckstein) . . . . .	348	The basis of chemotherapy (Thomas S. Work and Elizabeth Work) List of species maintained in the National Collection of Type Cultures . . . . .	658
Bergey's Manual of determinative bacteriology, 6th ed. (Robert S. Breed, E. G. D. Murray and A. Parker Hitchens) . . . . .	348		659
Endocrinology of neoplastic diseases: a symposium by 18 authors (Ed. by Gray H. Twombly and George T. Pack) . . . . .	349	SALICYLATE, effects on foetus and mother . . . . .	587
Identification of tumors (N. Chandler Foot) . . . . .	350	<i>Salmonella chittagong</i> : a new type of <i>Salmonella</i> . . . . .	35
	351	SARCOMA of heart . . . . .	344
		SEBACEOUS GLAND in cervix uteri . . . . .	511



	PAGE		PAGE
SEMEN, leucocytes and bacteria in . . .	131	TECHNIQUE, HISTOLOGICAL: demon-	
SERA: agglutination of coliform		stration of glycogen in necropsy	
bacilli by normal rabbit sera . . .	307	material . . . . .	325
polarographic analysis of patho-		demonstration of reticulo-endothelial	
logical sera . . . . .	152	cells in paraffin sections . . . .	515
SIDEROCYTES in blood of normal and		gastric mucosa found in aspirated	
premature infants . . . . .	342	resting juice . . . . .	333
SKIN, melanin-forming epidermal		marrow biopsy in rabbits . . . .	332
tumours of . . . . .	21	preparation of small tissue fragments	
STAPHYLOCOCCI, pathogenic, in throat:		for histology . . . . .	633
their incidence, especially in		TEMPERATURE: its effect on antibody	
glandular fever . . . . .	157	production in cold-blooded verte-	
STAPHYLOCOCCUS, effect of NaCl on		brates . . . . .	87
culture of . . . . .	553	THROMBOSIS as a factor in the patho-	
STATUS ASTHMATICUS, acute parietal		genesis of aortic atherosclerosis .	57
endocarditis in . . . . .	621	THYROID TUMOURS . . . . .	295
STILBÆSTROL: squamous metaplasia		TOXIN, diphtheria, mechanism of	
in prostate as result of stilbæstrol		parenchymatous degeneration pro-	
therapy . . . . .	330	duced by . . . . .	75
STOMACH: gastric mucosa found in		production by <i>C. diphtheriae</i> . . .	595
aspirated resting juice . . . . .	333	<i>Treponema pallidum</i> : its cultivation	
STREPTOCOCCI, canine beta hæmolytic		in tissue culture . . . . .	339
group-B, immunological studies with		TRILOCULAR HEART . . . . .	379
hæmolytic: anti-hæmolytic action of		<i>Trypanosoma congolense</i> , chemotherapy	
<i>viridans</i> ( $\alpha$ ) variants of hæmolytic		with phenanthridinium compounds	
streptococci . . . . .	199	TUBERCULOSIS in a wild sparrow-hawk	583
TECHNIQUE, BACTERIOLOGICAL: cata-		TUMOURS, ANIMAL: sarcoma of tibia	
lase production by Gram-positive		in a coypu rat . . . . .	154
cocci: a simple test for differentiat-		seminoma of testis in a collared turtle	
ing enterococci from micrococci .	135	dove . . . . .	155
clotting of human citrated plasma		TUMOURS, HUMAN: adamantinoma of	
by Gram-negative organisms . . .	247	jaw with pulmonary metastases .	193
cultivation of <i>T. pallidum</i> in tissue		large-cell, small-acinar thyroid	
culture . . . . .	339	tumour of Langhans . . . . .	295
rapid identification of <i>Proteus</i> .	183	liposarcoma arising in simple lipoma	483
selective medium for isolating		melanin-forming epidermal tumours	
<i>Staphylococcus</i> based on differ-		of skin . . . . .	21
ential inhibiting effect of NaCl .	553	sarcoma of heart . . . . .	344
simple method for testing dye		UREASE REACTIONS of <i>Proteus</i> group .	171
sensitivity of <i>Brucella</i> species .	328	UTERUS: sebaceous gland in cervix .	511
urease and other biochemical reac-			
tions in the <i>Proteus</i> group . . .	171		

# INDEX OF AUTHORS

	PAGE		PAGE
ABUL-FADL, M. A. M., and E. J. KING, application of formaldehyde to determination of serum acid phosphatase . . . . .	149	BRAY, J., and T. E. D. BEAVAN, slide agglutination of <i>Bacterium coli</i> var. <i>neapolitanum</i> in summer diarrhœa . . . . .	395
ADAMSON, H., C. H. BROWNING and K. M. CALVER, chemotherapy of <i>Trypanosoma congolense</i> infection with phenanthridinium compounds etc. . . . .	336	BROWNING, C. H., K. M. CALVER and H. ADAMSON, chemotherapy of <i>Trypanosoma congolense</i> infection with phenanthridinium compounds etc. . . . .	336
ADAMSON, W. W., primary sarcoma of heart . . . . .	344	BUCKLE, G., P. MACCALLUM, J. C. TOLHURST and H. A. SISSONS, a new mycobacterial infection in man . . . . .	93
AMIES, C. R., bacterial variation and toxin production by <i>Corynebacterium diphtheriæ</i> . . . . .	595	BYROM, F. B., and L. F. DODSON, causation of acute arterial necrosis in hypertensive disease . . . . .	357
ANDERSON, E. S., J. TAYLOR, W. HAYES and J. FREEMAN, new <i>Salmonella</i> type: <i>Salmonella chittagong</i> . . . . .	35	CALVER, K. M., C. H. BROWNING and H. ADAMSON, chemotherapy of <i>Trypanosoma congolense</i> infection with phenanthridinium compounds etc. . . . .	336
ARMSTRONG, E. C., and G. M. BONSER, effect of castration upon the induction of mammary tumours by œstrogen in male mice of the Strong A strain . . . . .	517	CAMERON, G. R., G. H. COORAY and S. N. DE, effect of castration on the action of some barbiturates . . . . .	239
BALASUBRAHMANYAN, M., and E. W. GAULT, cerebral cysticercosis . . . . .	505	CAMPBELL, A. C. P., the bone marrow in glandular fever . . . . .	629
BARAKAN, T. H., G. R. BARKER, J. M. GULLAND and L. D. PARSONS, tissue changes in mice treated with pentose nucleotides and related compounds . . . . .	441	CAMPBELL, A. C. P., histological preparation of bone-marrow particles, uterine curettings and other small tissue fragments . . . . .	633
BARKER, G. R., T. H. BARAKAN, J. M. GULLAND and L. D. PARSONS, tissue changes in mice treated with pentose nucleotides and related compounds . . . . .	441	CAMPBELL, A. C. P., incidence of pathogenic staphylococci in the throat, with special reference to glandular fever . . . . .	157
BARSON, G. J., and J. F. D. SHREWSBURY, intestinal flora of <i>Blatella americana</i> . . . . .	506	COLLINS, D. H., bullet embolism: pulmonary embolism following entry of a bullet into the right ventricle of the heart . . . . .	205
BARSON, G. J., and J. F. D. SHREWSBURY, intestinal flora of <i>Melolontha vulgaris</i> . . . . .	510	COLLINS, D. H., and W. McI. ROSE, nature of anæmia in leukæmia . . . . .	63
BEAVAN, T. E. D., and J. BRAY, slide agglutination of <i>Bacterium coli</i> var. <i>neapolitanum</i> in summer diarrhœa . . . . .	395	CONWAY, N. S., and E. M. HARPER, clotting of human citrated plasma by Gram-negative organisms . . . . .	247
BISSET, K. A., effect of temperature on antibody production in cold-blooded vertebrates . . . . .	87	COOK, G. T., urease and other biochemical reactions of <i>Proteus</i> group . . . . .	171
BOLTJES, T. Y. K., function and arrangement of bacterial flagella . . . . .	275	COORAY, G. H., G. R. CAMERON and S. N. DE, effect of castration on the action of some barbiturates . . . . .	239
BONSER, G. M., and E. C. ARMSTRONG, effect of castration upon the induction of mammary tumours by œstrogen in male mice of the Strong A strain . . . . .	517	COORAY, G. H., and J. C. HAWKESLEY, observations on fragments of gastric mucous membrane found in aspirated resting gastric juice . . . . .	333
BONSER, G. M., and M. J. STEWART, melanin-forming epidermal tumours of skin: a study of 57 personally observed cases . . . . .	21	CRUICKSHANK, A. H., bronchiectasis in laboratory rats . . . . .	520
	665	CRUICKSHANK, J. C., simple method for testing dye sensitivity of <i>Brucella</i> species . . . . .	328

	PAGE		PAGE
CUNNINGHAM, G. J., trilocular heart with bilateral aneurysmal dilatation of the pulmonary arteries . . . . .	379	GORDON, M., W. GOLDIE and K. I. JOHNSTONE, mutation in a single-cell culture of <i>Corynebacterium diphtheriae</i> . . . . .	369
DAVIDSON, J. I., massive colony formation of <i>Bacterium friedländeri</i> in liver in agranulocytosis . . . . .	51	GULAND, J. M., T. H. BAJAKAN, G. R. BARKER and L. D. PARSONS, tissue changes in mice treated with pentose nucleotides and related compounds . . . . .	441
DAVIDSON, J. N., I. LESLIE and J. C. WHITE, the cytoplasmic basophilia of marrow cells: distribution of nucleic acids . . . . .	1	HARPER, E. M., and N. S. CONWAY, clotting of human citrated plasma by Gram-negative organisms . . . . .	247
DAY, T. D., the spread of fluids in connective tissue . . . . .	150	HARRISON, C. V., experimental pulmonary arteriosclerosis . . . . .	289
DE, S. N., G. R. CAMERON and G. H. COORAY, effect of castration on the action of some barbiturates . . . . .	239	HARRISON, C. V., E. J. KING and G. NAGELSCHMIDT, effects of kaolin on the lungs of rats . . . . .	435
DISCOMBE, G., quantitative description of fragility of the erythrocyte and its application to the study of acholuric jaundice . . . . .	315	HARRISON, J. M., tuberculosis in a wild sparrow-hawk ( <i>Accipiter nisus</i> ) . . . . .	583
DODSON, L. F., and F. B. BYROM, causation of acute arterial necrosis in hypertensive disease . . . . .	357	HAWKSLEY, J. C., and G. H. COORAY, observations on fragments of mucous membrane found in aspirated resting gastric juice . . . . .	333
DOUGHERTY, C. M., a sebaceous gland in the cervix uteri . . . . .	511	HAYES, W., J. TAYLOR, J. FREEMAN and E. S. ANDERSON, new <i>Salmonella</i> type, <i>Salmonella chittagong</i> . . . . .	35
DUGUID, J. B., thrombosis as a factor in the pathogenesis of aortic atherosclerosis . . . . .	57	HENDERSON, W., some of the factors concerned in intracutaneous injection of cattle . . . . .	137
DUGUID, J. P., influence of cultural conditions on morphology of <i>Bacterium aerogenes</i> , with reference to nuclear bodies and capsule size . . . . .	265	HUMPHREY, J. H., pathogenesis of glomerulo-nephritis: a reinvestigation of the auto-immunisation hypothesis . . . . .	211
EDMONDS, D. G., and H. P. WRIGHT, siderocyte counts in the blood of normal and premature infants . . . . .	342	INGLIS, A., squamous metaplasia in prostate as a result of stilbæstrol therapy . . . . .	330
ELEK, S. D., rapid identification of <i>Proteus</i> . . . . .	183	INGLIS, K., obituary notice of David Arthur Welsh . . . . .	638
EMSLIE-SMITH, A. H., agglutination of coliform bacilli by normal rabbit serum . . . . .	307	ISAACS, A., anti-hæmolytic action of <i>viridans</i> ( $\alpha$ ) variants of hæmolytic streptococci . . . . .	199
EVANS, R. W., antibody response in radiation lymphopenia and the reticuloses . . . . .	123	ISAACS, A., and J. M. SCOLLER, catalase production by Gram-positive cocci: a simple test for differentiating enterococci from micrococci . . . . .	135
FENNER, F., clinical features and pathogenesis of mouse-pox (infectious ectromelia of mice) . . . . .	529	JACKSON, A. V., toxic effects of salicylate on the fœtus and mother . . . . .	587
FREEMAN, J., J. TAYLOR, W. HAYES and E. S. ANDERSON, new <i>Salmonella</i> type: <i>Salmonella chittagong</i> . . . . .	35	JEBB, W. H. H., starch-fermenting gelatin-liquefying corynebacteria isolated from the human nose and throat . . . . .	403
GAULT, E. W., and M. BALASUBRAHMANYAN, cerebral cysticercosis . . . . .	505	JOHNSTONE, K. I., W. GOLDIE and M. GORDON, mutation in a single-cell culture of <i>Corynebacterium diphtheriae</i> . . . . .	369
GINSBERG, A., and A. C. W. LITTLE, actinomycosis in dogs . . . . .	563	KING, E. J., and M. A. M. ABUL-FADEL, application of formaldehyde to determination of serum acid phosphatase . . . . .	149
GOLDIE, W., M. GORDON and K. I. JOHNSTONE, mutation in a single-cell culture of <i>Corynebacterium diphtheriae</i> . . . . .	369		
GORDON, J., a non-gas-producing variant of <i>Bacterium coli</i> induced by growth in glycine . . . . .	521		

# INDEX OF AUTHORS

	PAGE		PAGE
KING, E. J., C. V. HARRISON and G. NAGELSCHEIDT, effects of kaolin on the lungs of rats . . . . .	435	ORR, J. W., D. E. PRICE and L. H. STICKLAND, glycogen content of rats' livers after poisoning with large doses of <i>p</i> -dimethylamino-azobenzene . . . . .	573
LAUGHTON, N., canine beta hæmolytic streptococci . . . . .	471	PARSONS, L. D., T. H. BARAKAN, G. R. BARKER and J. M. GULLAND, tissue changes in mice treated with pentose nucleotides and related compounds . . . . .	441
LENNOX, B., acute parietal endocarditis in a case of status asthmaticus: a possible early stage of Löffler's endocarditis parietalis fibroplastica with eosinophilia . . . . .	621	PATTISON, I. H., immunological studies with group-B streptococci . . . . .	219
LENNOX, B., large-cell small-acinar thyroid tumour of Langhans and the incidence of related cell groups in the human thyroid . . . . .	295	PERRY, W. L. M., cultivation of <i>Treponema pallidum</i> in tissue culture . . . . .	339
LESLIE, I., J. N. DAVIDSON and J. C. WHITE, the cytoplasmic basophilia of marrow cells: distribution of nucleic acids . . . . .	1	POPJÁK, G., mechanism of parenchymatous degeneration produced by diphtheria toxin . . . . .	75
LITTLE, A., and A. GINSBERG, actinomycosis in dogs . . . . .	563	PRICE, D. E., and J. W. ORR, observations on the hepatotoxic action of the carcinogen <i>p</i> -dimethylamino-azobenzene . . . . .	461
MACCALLUM, P., J. C. TOLHURST, G. BUCKLE and H. A. SISSONS, a new mycobacterial infection in man . . . . .	93	PRICE, D. E., J. W. ORR and L. H. STICKLAND, glycogen content of rats' livers after poisoning with large doses of <i>p</i> -dimethylamino-azobenzene . . . . .	573
McFADZEAN, A. J. S., marrow biopsy in laboratory animals . . . . .	332	PRYCE, D. M., lining of healed but persistent abscess cavities in lungs with epithelium of ciliated columnar type . . . . .	259
MAGAUAN, W., and A. G. RICKARDS, Fallopian tube granuloma . . . . .	512	PULLAR, T. H., and G. E. WATERWORTH, adamantinoma of jaw with pulmonary metastases . . . . .	193
MATTLAND, H. B., and G. MARTYN, selective medium for isolating <i>Staphylococcus</i> based on the differential inhibiting effect of increased concentrations of sodium chloride . . . . .	553	RAEBURN, C., idiopathic ("isolated") myocarditis in infancy . . . . .	477
MARSHALL, A. H. E., method for demonstration of reticulo-endothelial cells in paraffin sections . . . . .	515	REWELL, R. E., perforation of palate and destruction of nasal bones due to nocardiosis in a Bennett's wallaby ( <i>Macropus rufogrisea</i> ) . . . . .	155
MARTYN, G., and H. B. MATTLAND, selective medium for isolating <i>Staphylococcus</i> based on the differential inhibiting effect of increased concentrations of sodium chloride . . . . .	413	REWELL, R. E., sarcoma of tibia in a coypu rat ( <i>Myocastor coypus</i> ) . . . . .	154
MOLE, R. H., fibrinolysin and the fluidity of the blood <i>post mortem</i> . . . . .	354	REWELL, R. E., seminoma of testis in a collared turtle dove ( <i>Streptopelia risoria</i> ) . . . . .	155
MORISON, J. E., neoprene latex casts in the study of lung development . . . . .	638	RICKARDS, A. G., and W. MAGAUAN, Fallopian tube granuloma . . . . .	512
MUIR, R., obituary notice of David Arthur Welsh . . . . .	429	ROBERTSON, J. S., and K. V. SMITH, analysis of certain factors associated with the production of aortic media, in relation to the pathogenesis of dissecting aneurysm . . . . .	43
MULDER, J., and M. STRAUB, epithelial lesions in the respiratory tract in human influenzal pneumonia . . . . .	435	ROBINSON, A. M., and F. L. WARREN, use of polarographic analysis in the investigation of pathological sera . . . . .	152
NAGELSCHEIDT, G., E. J. KING and C. V. HARRISON, effects of kaolin on the lungs of rats . . . . .	435	ROSE, W. McI., and D. H. COLLINS, nature of anæmia in leukaemia . . . . .	63
OAKLEY, C. L., G. H. WARRACK and M. E. WARREN, kappa and lambda antigens of <i>Clostridium welchii</i> . . . . .	495	SCOLLER, J. M., and A. ISAACS, catalase production by Gram-positive cocci: a simple test for differentiating enterococci from micrococci . . . . .	461
ORR, J. W., and D. E. PRICE, observations on the hepatotoxic action of the carcinogen <i>p</i> -dimethylamino-azobenzene . . . . .	461		

	PAGE		PAGE
SENEVIRATNE, R. D., redistribution of body fluids in dysenteric toxæmia	609	TAYLOR, J., W. HAYES, J. FREEMAN and E. S. ANDERSON, new <i>Salmonella</i> type: <i>Salmonella chittagong</i>	35
SHREWSBURY, J. F. D., and G. J. BANSON, intestinal flora of <i>Blattella americana</i>	506	THOMSON, J. G., hæmolytic in fresh blood films	140
SHREWSBURY, J. F. D., and G. J. BANSON, intestinal flora of <i>Melolontha vulgaris</i>	510	TOLHURST, J. C., P. MACCALLUM, G. BUCKLE and H. A. SISSONS, a new mycobacterial infection in man	93
ŠIKL, H., Addison's disease due to congenital hypoplasia of adrenals in an infant aged 33 days	323		
SISSONS, H. A., P. MACCALLUM, J. C. TOLHURST and G. BUCKLE, a new mycobacterial infection in man	93	VALLANCE-OWEN, J., histological demonstration of glycogen in necropsy material	325
SMITH, J. F., disseminated focal necrosis with eosinophilia and arteritis in asthma (? Loeffler's syndrome)	489		
SMITH, K. V., and J. S. ROBERTSON, analysis of certain factors associated with the production of experimental dissection of the aortic media, in relation to the pathogenesis of dissecting aneurysm	43	WALTON, K., and A. G. SPENCER, Ebstein's anomaly of the tricuspid valve	387
SOLTYS, M. A., anthrax in a laboratory worker, with observations on the possible source of infection	253	WARRACK, G. H., C. L. OAKLEY and M. E. WARREN, kappa and lambda antigens of <i>Clostridium welchii</i>	495
SPENCER, A. G., and K. WALTON, Ebstein's anomaly of the tricuspid valve	387	WARREN, F. L., and A. M. ROBINSON, use of polarographic analysis in the investigation of pathological sera	152
STEWART, M. J., and G. M. BONSER, melanin-forming epidermal tumours of skin: a study of 57 personally observed cases	21	WARREN, M. E., C. L. OAKLEY and G. H. WARRACK, kappa and lambda antigens of <i>Clostridium welchii</i>	495
STICKLAND, L. H., J. W. ORR and D. E. PRICE, glycogen content of rats' livers after poisoning with large doses of <i>p</i> -dimethylaminoazobenzene	573	WATERWORTH, G. E., and T. H. PULLAR, adamantinoma of jaw with pulmonary metastases	193
STRAUB, M., and J. MULDER, experimental lesions in the respiratory tract in human influenzal pneumonia	429	WHITBY, L. E. H., obituary notice of Edward ffolliott Creed	635
SVENDSEN, M., leucocytes and bacteria in human semen	131	WHITE, J. C., J. N. DAVIDSON and I. LESLIE, the cytoplasmic basophilic of marrow cells; distribution of nucleic acids	1
		WRIGHT, C. J. E., liposarcoma arising in a simple lipoma	483
		WRIGHT, H. P., and D. G. EDMONDS, siderocyte counts in the blood of normal and premature infants	342

